UNITED STATES PATENT APPLICATION

OF

Charles E. Hart, Richard D. Kenagy and Alexander W. Clowes

FOR

INHIBITION OF INTIMAL HYPERPLASIA USING ANTIBODIES TO PDGF
RECEPTORS

Description

5   INHIBITION OF INTIMAL HYPERPLASIA USING ANTIBODIES TO
PDGF RECEPTORS

Cross-Reference to Related Applications

    This application is a continuation-in-part of
10 U.S. Patent Application Serial No. 08/366,860, filed
December 30, 1994, which application is pending and which
is a continuation-in-part of U.S. Patent Application
Serial No. 08/304,623, filed September 12, 1994 and now
abandoned, which application is a continuation of Serial
15 No. 08/023,504, filed February 25, 1993 and now
abandoned, and which are incorporated herein by reference
in their entirety.

Government Support

20   This invention was made with government support
under grant number NIH HL 30946 awarded by the National
Institutes of Health. The government has certain rights
in the invention.

25 Technical Field

    The present invention relates to methods for
inhibiting intimal hyperplasia, including restenosis, in
a mammal following vascular injury, and to compositions
useful within those methods.

30

Background of the Invention

    Proliferation of smooth muscle cells (SMCs) in
the vessel wall is an important event in the formation of
vascular lesions in atherosclerosis, after vascular
35 reconstruction or in response to other vascular injury.
For example, treatment of atherosclerosis frequently
includes the clearing of blocked vessels by angioplasty,

endarterectomy or reducti n atherectomy, or by bypass grafting, surgical procedures in which atherosclerotic plaques are compressed or removed through catheterizati n (angioplasty), stripped away from the arterial wall through an incision (endarterectomy) or bypassed with natural or synthetic grafts. These procedures remov the vascular endothelium, disturb the underlying intimal layer, and result in the death of medial SMCs. This injury is followed by medial SMC proliferation and migration into the intima, accompanied by excessive deposition of extracellular matrix. This lesi n development characteristically occurs within the first few weeks and up to six months after injury and stops when the overlying endothelial layer is reestablished. In humans, these lesions are composed of about 20% cells and 80% extracellular matrix.

In about 30% or more of patients treated by angioplasty, endarterectomy or bypass grafts, thrombosis and/or SMC proliferation in the intima causes re-occlusion of the vessel and consequent failure of the reconstructive surgery. This closure of the vess l subsequent to surgery is known as restenosis.

A similar process of SMC proliferation has also been observed in organ transplants, and may contribute to transplant atherosclerosis and organ failure. Th intimal thickening in this process involves only the grafted organ.

It has been postulated that platelet mitogens, such as platelet derived growth factor (PDGF), play a role in the development of atherosclerotic plaques (see Ross et al., Cell 46: 155-169, 1986; Harker, Am. J. Cardiol. 60: 20B-28B, 1987). One proposed mechanism for plaque formation is the release by platelets, at sites of endothelial denudation, of growth factors that stimulate SMC gr wth (R ss and G!omset, N. Eng. J. Med. 295: 369-377, 420-425, 1976; R ss, Arteriosclerosis 1: 293-311, 1981). Moore et al. (Thrombos. Haemostas. (Stuttg.) 35:

70, 1976) and Friedman et al. (*J. Clin. Invest.* 60: 1191-1201, 1977), using an indw lling catheter injury model, reported an inhibition of experimentally induced intimal lesion formation in rabbit arteries by prolonged thrombocytopenia induced by administration of anti-platelet serum. It has also been postulated that SMCs may themselves produce PDGF which stimulates lesion development through an autocrine mechanism (Ross et al., ibid; Walker et al., *Proc. Natl. Acad. Sci. USA* 83: 7311-7315, 1986). Fingerle et al. (*Proc. Natl. Acad. Sci. USA* 86: 8412-8416, 1989) investigated intimal lesion formation in thrombocytopenic rats and concluded that platelets do not play a role in the initial SMC proliferation after balloon injury but may regulate SMC migration into the intima. Platelets are now known to release a number of growth factors, including PDGF, epidermal growth factor (EGF), transforming growth factors alpha and beta (TGFα and TGFβ), insulin-like growth factor I (IGF-I) and platelet derived endothelial cell growth factor, as well as several chemoattractant molecules. Although certain studies implicate PDGF in processes associated with lesion development, no studies have shown the participation of PDGF in these processes in primates.

Removal of atherosclerotic plaques by angioplasty or endarterectomy has limited efficacy, and no effective treatment for restenosis of treated vessels or stenosis of bypass grafts has been developed. There is therefore a need in the art for methods of reducing or preventing the development of SMC-rich lesions in vascular walls, including stenosis of blood vessels following vascular injury, such as injury due to balloon catheterization, endarterectomy or reduction atherectomy, as well as in vascular grafts, organ transplants and catheter emplac ments. The present inventi n pr vides such methods and fulfills other, related needs.

Disclosure of the Invention

Th present inventi n pr vides methods and compositions for inhibiting intimal hyperplasia in the vasculature of a mammal, particularly a primate. Examples of intimal hyperplasia include resten sis following angioplasty, endarterectomy or other procedures whereby atherosclerotic plaques are removed from blood vessels. Within one aspect of the invention the methods generally comprise administering to a mammal an effective amount of an anti-growth factor receptor antibody t inhibit intimal hyperplasia. Suitable anti-growth factor receptor antibodies include antibodies to fibroblast growth factor (FGF) receptors, transforming growth factor beta (TGF-β) receptors, insulin-like growth factor I (IGF-I) receptors, epidermal growth factor (EGF) receptors, thrombin receptors and factor Xa receptors.

It is preferred within the present invention to utilize an anti-platelet derived growth factor (PDGF) receptor antibody in an amount sufficient to inhibit mitogenesis and/or migration of smooth muscle cells. An anti-PDGF receptor antibody may be administered alone, in combination with other anti-PDGF receptor antibodies, in combination with antibodies to other receptors, or in combination with a heparin.

Within one aspect of the invention, intimal hyperplasia is inhibited by administering to a mammal an effective amount of an anti-PDGF receptor antibody, such as an anti-PDGF-alpha receptor antibody, an anti-PDGF-beta receptor antibody, or a panel of anti-PDGF receptor antibodies. Within one embodiment, the panel of antibodies is capable of inhibiting the binding of the AA, AB and BB isoforms of PDGF to PDGF receptors.

Within another aspect of the invention, an anti-PDGF receptor antibody is administered to a mammal c ncurrently with, or within an antihyperplastically eff ctive time period prior to, an occurrance of an acute vascular injury in the mammal. Examples of acut

vascular injuries includ vascular reconstruction procedur s such as angioplasty, endarterect my, reduction atherectomy and anastomosis of a vascular graft. In a related aspect, the antibcdy is administered concurrently with, or within an antihyperplastically effective time period prior to, emplacement of a vascular graft or transplanted organ. Within other embodiments, the antibody is administered within an antihyperplastically effective time period following an occurance of an acute vacular injury or emplacement of a vascular graft or transplanted organ.

Within another aspect of the invention, the antibodies are used to inhibit intimal hyperplasia that occurs within a vascular graft or transplanted organ.

Within another aspect of the invention, intimal hyperplasia in the vasculature of a mammal is inhibited by coordinately administering to the mammal a PDGF antagonist and heparin in respective amounts of antagonist and heparin sufficient to combinatorially inhibit the hyperplasia. The PDGF antagonist and heparin are administered concurrently or, alternatively, sequentially with either the antagonist or heparin administered first, and the nonadministered remainder f the antagonist and heparin administered within an effective time period thereafter. Within one embodiment, the PDGF antagonist is a non-peptidic PDGF antagonist. Wtihin another embodiment, the PDGF antagonist is an anti-growth factor receptor antibody, such as an anti-PDGF receptor antibody,

Within related embodiments, the coordinately administered antibody or other PDGF antagonist and heparin combinatorially inhibit one or more of the intimal hyperplastic processes of vascular smooth muscle cell proliferation, vascular smooth muscle cell migrati n, and/or n ointimal deposition of extracellular matrix. Within further embodiments, the antibody is either an anti-PDGF-alpha recept r antibody, an

anti-PDGF-beta r cept r antibody, or a panel f anti-PDGF rec pt r antibodies. Within additi nal embodiments, the antibody or other PDGF antagonist inhibits a recept r function of the growth factor receptor, such as binding
5 of the receptor to a receptor ligand, or dimerizati n of the growth factor receptor. Within other embodiments, an anti-PDGF antibody or other PDGF antagonist is administered which inhibits binding of one or more of the AA, AB and BB isoforms of PDGF to PDGF receptors. Within
10 other embodiments, the heparin comprises a h paran sulfate or a low molecular weight heparin characterized by having a reduced anti-thrombotic activity.

Within other aspects of the invention, an anti-PDGF receptor antibody or other PDGF antagonist is
15 coordinately administered with heparin to a mammal concurrently with, or within an effective time p riod before, an occurrence of an acute vascular injury in the mammal. Acute vascular injuries include vascular injuries arising from vascular reconstruction, including
20 injuries due to angioplasty, endovascular stenting, endarterectomy, endovascular laser ablation, reducti n atherectomy or anastomosis of a vascular graft. In related aspects, a PDGF antagonist and heparin are administered concurrently with, or within a
25 therapeutically effective time period before, emplacement of a vascular graft or transplanted organ. Within ther embodiments, a PDGF antagonist and heparin ar administered within an antihyperplastically effectiv time period following an occurance of an acute vacular
30 injury or emplacement of a vascular graft or transplanted organ.

Within yet another aspect of the invention, pharmaceutical kits are provided for the treatm nt of intimal hyperplasia in a mammal, which kits include a
35 PDGF antagonist, such as an anti-PDGF recept r antibody r a non-peptidic PDGF antag nist, and heparin in a pharmac logically suitable carrier. In one embodiment,

the antag nist and heparin are pre-combined in a single carrier. In another embodiment, the antag nist and heparin are administrable by simultaneous, separate or sequential delivery.

5   Antibodies useful within the present inventi n include monoclonal antibodies and genetically engineered antibodies, the latter including single chain antibodies, chimeric antibodies, bifunctional antibodies and immunoconjugates.

10   Heparin preparations useful within the pres nt invention include unfractionated or fractionated heparins and heparin-like glycosaminoglycans, including heparan sulfates. Also useful are low molecular weight heparins, including anticoagulant and nonanticoagulant fragments

15 and derivatives of heparin and heparin-like glycosaminoglycans.

          These and other aspects of the invention will become evident upon reference to the following detailed description and the attached drawings.

20

Brief Description of the Drawings

          Figure 1 illustrates the binding of anti-PDGF receptor monoclonal antibodies to cells that express recombinant PDGF-beta receptor. Results are expressed as

25 mean cpm bound of ^{125}I-rabbit anti-mouse IgG for triplicate determinations. The bars indicate standard deviation.

          Figure 2 illustrates the binding of anti-PDGF receptor monoclonal antibodies to cells that express

30 recombinant PDGF-alpha receptor. Results are express d as mean cpm bound of ^{125}I-rabbit anti-mouse IgG for triplicate determinations. The bars indicate standard deviation.

          Figures 3A-3C illustrate the inhibition of PDGF

35 mit genic activity on human dermal fibr blasts by anti-PDGF recept r m n clonal antibodies. Th results are presented as the mean level of [^{3}H]thymidine

incorp ration for each of th PDGF ligand test conditi ns. Standard d viation is shown by the T at the top of each bar. Each panel also shows a standard curve for PDGF ligand alone. A) PDGF-AA stimulation, B) PDGF-AB stimulation, C) PDGF-BB stimulation.

Figure 4 illustrates the inhibition of PDGF-AA mitogenic activity on baboon smooth muscle cells by anti-PDGF receptor monoclonal antibodies. A standard curve for ligand alone is shown on the left. Result are presented as the mean level of [^{3}H] thymidine incorporation. Standard deviation is shown by the T at the top of each bar.

Figure 5 illustrates the inhibition of PDGF-AB mitogenic activity on baboon smooth muscle cells by anti-PDGF receptor monoclonal antibodies. A standard curve for ligand alone is shown on the left. Results are presented as in Figure 4.

Figure 6 illustrates the inhibition of PDGF-BB mitogenic activity on baboon smooth muscle cells by anti-PDGF receptor monoclonal antibodies. A standard curve for ligand alone is shown on the left. Results are presented as in Figure 4.

Figures 7 A and B illustrate titrati n f representative monoclonal antibodies to inhibit the mitogenic activity of PDGF-AA on baboon smooth muscle cells. The results are presented as the mean level of [^{3}H] thymidine incorporation for each of the PDGF-AA test conditions. Standard deviation is shown by the T for the PDGF-AA standard curve samples. (A) Standard curve of PDGF-AA mitogenic activity. (B) Inhibitory potency of MAbs 169.14 and 169.31 for PDGF-AA mitogenic activity as shown by a decrease in the level of [^{3}H] thymidine incorporation.

Figure 8 illustrates the inhibition of babo n serum mitogenic activity on bab on sm oth muscle cells by anti-PDGF receptor mon clonal antibodi s. A standard curve f r serum alone is shown on the left. The results

are present d as the mean level of [^{3}H] thymidine
incorporation. Standard deviation is shown by the T at
the top of each bar.

Figure 9 illustrates the mitogenic activity of
5 baboon serum, in the presence of anti-PDGF alpha receptor
monoclonal antibody 169.31, on baboon smooth muscle
cells.

Detailed Description of the Invention

10   As noted above, restenosis of blood vessels is
a common problem in patients who have undergone
angioplasty, endartarectomy, or bypass grafting.
Restenosis is one example of intimal hyperplasia, which
is believed to proceed via a process that includes both
15 proliferation (mitosis) and migration of vascular smooth
muscle cells in the area damaged by the surgical
procedure, as well as by the production (deposition) of
extracellular matrix. See, in general, (Harker, Am. J.
Cardiol. 60:20B-28B, 1987; DeFeudis, Drug News and
20 Perspectives 5:49-51, 1992). This proliferative process
is also manifested in the occlusion of vascular grafts
(both natural, including autologous and allogeneic, and
synthetic), and in transplanted organs. This
proliferative process results in the development of
25 lesions rich in smooth muscle cells and is refered to
herein as intimal hyperplasia.

The present invention provides methods for
inhibiting the development of SMC-rich lesions through
the use of antibodies against growth factor receptors,
30 preferably PDGF receptors, and through the use of other
PDGF antagonists, particularly non-peptidic PDGF
antagonists. The term "non-peptidic" refers to compounds
other than proteins or other peptide-bonded multimers.
SMC-rich lesions result in the partial or complete
35 bl cking f a blood vessel through intimal thick ning
(hyperplasia). Inhibiti n of intimal hyperplasia will b
understood to include interfering with the proliferative

process by reducing r preventing ne r m re hyperplastic processes, including cell migration, cell proliferation, and extracellular matrix formation. By blocking proliferation and/or migration thr ugh interfering with the interaction of PDGF and its receptors, SMC proliferation and subsequent matrix deposition may be reduced. A reduction in intimal hyperplasia is clinically manifested as a significant decrease in loss of lumenal volume after an acute vascular injury. Such a reduction will generally result in a decreased need for re-vascularization procedures (e.g., repeat angioplasty) at the site of the initial injury.

The methods of the present invention are particularly useful in the treatment of intimal hyperplasia due to acute vascular injury. Acute vascular injuries are those which occur rapidly (i.e. over days t months), in contrast to chronic vascular injuries (e.g. atherosclerosis) which develop over a lifetime. Acut vascular injuries often result from surgical procedures such as vascular reconstruction, wherein the techniques of angioplasty, endarterectomy, atherectomy, vascular graft emplacement or the like are employed. Hyperplasia may also occur as a delayed response in response t , e.g., graft emplacement or organ transplantation.

The present invention utilizes PDGF antagonists to inhibit intimal hyperplasia in the vasculature of a mammal. PDGF antagonists are advantageously used in combination with heparin. PDGF antagonists useful within the present invention include non-peptidic PDGF antagonists and peptidic PDGF antagonists, such as anti-PDGF receptor antibodies.

One group of non-peptidic PDGF antagonists includes c mp unds of formula I or II:

(I)

(II)
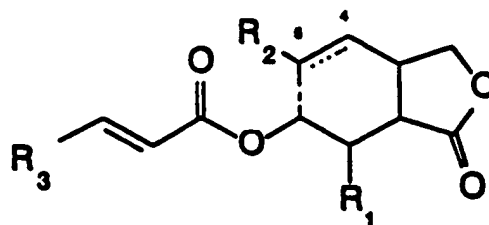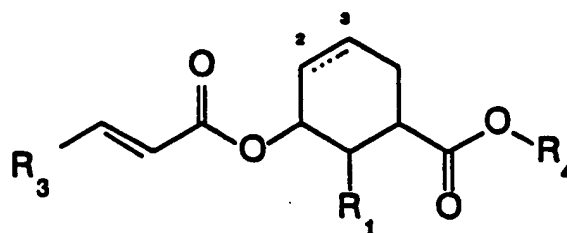
and pharmaceutically acceptable salts thereof. Within formulas I and II, the bond indicated by the solid and dotted line is single or double; R_1 is CO_2H or a carboxylic acid bioisostere; R_2 is H or a linear or branched, 1- to 6-carbon alkyl or alkoxy; R_3 is
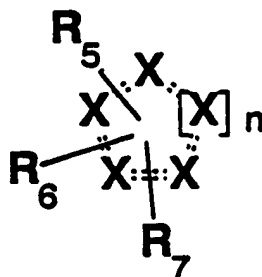
wherein n is 1 or 2, and each X is individually C, N, O, NH or S, with the proviso that at least two, preferably at least three, X are C; R_4 is H, a linear or branched alkyl or alkoxy of from 1-18 carbon atoms, or a mono-alkyl of not more than 6, preferably not more than 5, carbon atoms.

Preferred non-peptidic PDGF antagonists of this group include 92-0825:

, which has been f und
to inhibit binding of PDGF-BB to rat smooth muscle cells
and to inhibit PDGF-BB mitogenic activity on babo n
smooth muscle cells.

5   A second group of non-peptidic PDGF antag nists
includes   Brefeldin   A   (1,6,7,8,9,11a,12,13,14,14a-
Decahydro-1,13-dihydroxy-6-methyl-4H-
cyclopent[f]oxacyclotridecin-4-one)   and   derivativ s
thereof. Brefeldin A has the structure:

10
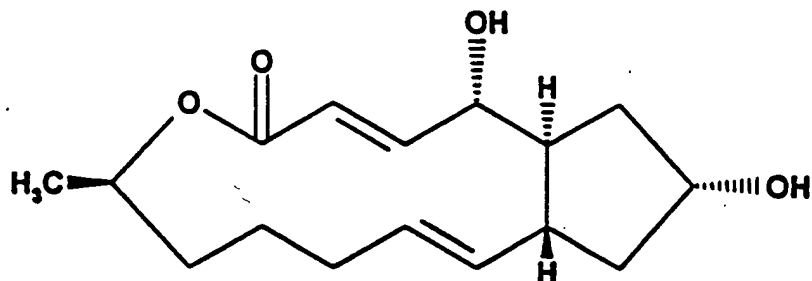
Brefeldin A has been found to inhibit, PDGF
mitogenic acitivity on baboon smooth muscle cells, which
acitivity is enhanced in the presence of heparin.

15   Antibodies useful within the present inventi n
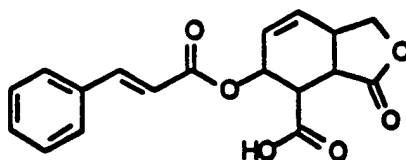may be produced by conventional procedures of
immunization and purification. Briefly, a PDGF recept r,
receptor fragment or fusion protein comprising a recept r
polypeptide, preferably purified, is administered to an
20   animal such as a mouse, rat, rabbit or goat in an amount
sufficient to cause an immune response. It is preferred
to administer the growth factor receptor in combination
with an adjuvant, such as Freund's adjuvant, in order to
enhance the immune response. Although a single injection
25   of antigen may be sufficient t induce antibody
production in the animal, it is generally preferr d to

administer a large initial injecti n f llowed by on or more booster injections over a period of several weeks to several months. See, e.g., Hurrell, J.G.R., ed., Monoclonal Hybridoma Antibodies: Techniques and
5 Applications, CRC Press Inc., Boca Raton, FL, 1982, which is incorporated herein by reference. Blood is then collected from the animal and clotted, and antibodies are isolated from the serum using conventional techniqu s such as salt precipitation, ion exchange chromatography,
10 affinity chromatography or high performance liquid chromatography.

Within one embodiment of the inventi n, monoclonal antibodies are used. Monoclonal antibodies provide the advantages of ease of production and l wer
15 therapeutic doses as compared to polyclonal antisera, since only antibodies of the desired specificity are used. Methods for producing monoclonal antibodies are well known in the art and are disclosed, for example, by Kohler and Milstein (Nature 256: 495, 1975; Eur. J.
20 Immunol. 6: 511-519, 1976). See also Hurrell, J.G.R., ed., Monoclonal Hybridoma Antibodies: Techniques and Applications, CRC Press Inc., Boca Raton, FL, 1982 and Hart, U.S. Patent No. 5,094,941. As will be appreciated by those skilled in the art, antibody fragments, such as
25 Fab fragments, may also be used.

It is generally preferred to use antibodi s that are syngenesious with the patient or that contain syngenesious constant regions. For this reason, genetically engineered antibodies that contain human
30 framework structures will generally be used in the treatment of humans. Methods for producing recombinant human antibodies or humanized non-human (i.e. chimeric) antibodies are disclosed by Cabilly et al. (U.S. Patent N . 4,816,567), Robinson et al. (WO 87/02671) and
35 Neumaier (WO 90/00616), which are inc rp rated herein by reference. Briefly, human constant region genes are join d to appr priate human or non-human variable region

gen *s*. For xampl , the amino acid sequences which represent the antigen binding sites (CDRs, or complimentarity-determining regions) of the parent murine monoclonal antibody are grafted at the DNA level onto human variable region framework sequences. This process is known as "humanization". Methods for this technique are known in the art and are disclosed, for example, by Jones et al. (Nature 326: 522-525, 1986), Riechmann et al. (Nature 322: 323-327, 1988) and Queen et al. (Proc. Natl. Acad. Sci. USA 86: 10029-10033, 1989).

The joined genes are then transfected into host cells, which are cultured according to conventi nal procedures. In the alternative, monoclonal antibody producing cells are transfected with cloned human constant region genes, and chimeric antibody genes are generated by homologous recombination. Thus it is possible to assemble monoclonal antibodies with a significant portion of the structure being human, thereby providing antibodies that are more suitable for multiple administrations to human patients.

Alternatively, a single chain antibody may be developed through the expression of a recombinant polypeptide which is generally composed of a variable light-chain sequence joined, typically via a linker polypeptide, to a variable heavy-chain sequence. M thods for producing single chain antibodies are known in the art and are disclosed, for example, by Davis et al. (BioTechnology 9: 165-169, 1991).

Two PDGF receptor polypeptides have been described. These are termed "alpha receptor" (Kelly et al., WO 90/14425; Kelly et al., U.S. Patent No. 5,371,205; Claesson-Welsh et al., Proc. Natl. Acad. Sci. USA 86: 4917-4921, 1989) and "beta receptor" (Claesson-Welsh et al., Mol. Cell. Biol. 8: 3476-3486, 1988; Gronwald et al., Proc. Natl. Acad. Sci. USA 85: 3435-3439, 1988). In the presence of PDGF ligand, the r ceptor polypeptides dimerize. Thre receptor subtypes

are thus possible: αα, αβ and ββ. The β r c pt r is specific for the B-chain f PDGF, while the α receptor binds the A-chain and the B-chain. Consequently, th growth regulatory responsiveness of cells to PDGF depends not only on the availability of PDGF AA, AB and BB ligand isoforms, but also on the expression and availability f different PDGF receptor subtypes (Heldin et al., _Cell Regul._ 1: 555-566, 1990). Human smooth muscle cells express both α and β receptor subtypes (Heldin et al., _Cell Regul._ 1: 555-566, 1990), but other cell types are known which express only a single receptor subtype (Gronwald et al., _J. Biol. Chem._ 264: 8120-8125, 1989).

The anti-PDGF receptor antibodies used within the present invention will preferably be a panel of antibodies capable of inhibiting all three PDGF recept r isoforms (αα, ββ and αβ). As used herein, the term "panel" denotes a combination of two or more antibodies having different specificities. The antibodies may be specific for different antigens or for different epitopes on a single antigen. Monoclonal antibodies (MAbs) are preferred.

As noted above, antibodies used within the present invention interfere with the interaction of PDGF and its receptors. In preferred embodiments of the invention, anti-PDGF receptor antibodies are employed which inhibit binding of a PDGF ligand to a PDGF receptor, although those skilled in the art will recognize that the advantages of the invention can also be realized using antibodies that inhibit other receptor-ligand interactions, such as receptor dimerization.

Anti-receptor monoclonal antibodies may also be used as targeting agents for the delivery of compounds of therapeutic interest. Such compounds include, but are not limited to, toxins, cytostatic compounds, or pr enzym s wh se potential functi n can be to activate endog n us pr enzymes, t activate pr enzymes added from exogen us s urces, or t activate enzyme cleavage sites

on prodrugs. Anti-recept r antibodies can also be labeled with radi nucle tid s, dyes, fluorescent compounds or the like for use as imaging agents. Examples of this include imaging sites of thrombosis, or sites of vascular injury where there is exposure, by example, of vascular smooth muscle cells which express cell-surface receptors.

Monoclonal antibodies can also be used to develop bifunctional antibodies where there are two independent antigenic binding sites on each immunoglobulin molecule. This technology is known in the art and has been disclosed in the literature (Thromb. Res. Suppl. X: 83, 1990). Additionally, bispecific antibodies can also be constructed from single chain antibodies. This technology is known in the art and has been disclosed, for example, by A. George (The Second Annual IBC International Conference on Antibody Engineering, Dec. 16-18, 1991, San Diego CA).

Antibodies used within the present inv ntion will be able to block a significant amount of the biological activity of an antigen in an *in vitro* t st system, e.g. the ability to block the interaction of one or more PDGF ligands with PDGF receptor(s). Suitabl *in vitro* test systems include, *inter alia*, mitogene is assays and receptor binding assays. For exampl , 25 $\mu g/ml$ of a monoclonal anti-PDGF-alpha receptor MAb described herein is able to block the mitogenic activity of 10 ng/ml of PDGF-AA. As will be understood by those skilled in the art, the amount of antibody needed t inhibit the activity of a given amount of antigen will depend on such factors as antibody specificity and affinity. It is preferred not to block 100% of serum mitogenic activity so that not all of the wound healing response is suppressed. Antibody doses are calculat d as described bel w, taking int consideration affinity and specific activity.

An "antihyperplastically effective am unt" of
an anti-PDGF recept r antibody r other PDGF antagonist
is defined as an amount sufficient to measurably reduce
or prevent intimal hyperplasia in a blood vessel, vessel
5　graft or vascular component of a transplanted organ.
More specifically, "inhibition of intimal hyperplasia" is
herein defined to include any measurable inhibition of
one or more of the intimal hyperplastic process s
described in the art as vascular smooth muscle cell
10　(VSMC) migration, VSMC proliferation, and neointimal
deposition of extracellular matrix. In this cont xt,
reduction or prevention of intimal hyperplasia, or of a
hyperplastic process involved in intimal hyperplasia, can
be readily evaluated using *in vitro, in vivo* and *ex vivo*
15　assay systems known in the art, in particular primate-
based assay systems (e.g., non-human or human primate
VSMC cultures or vascular tissue explants, or non-human
primate *in vivo* tests). In interpreting *in vitro* d sage
data, it will be appreciated that different test cells
20　and tissues may express different levels and/or types of
PDGF receptors. In addition, cell culture passage numb r
(i.e. number of cell generations elapsed foll wing
dissociation or outgrowth of VSMCs from a vascular tissu
source) will be recognized as potentially having an
25　important impact on mitogenic and other growth-r lated
activities observed in experimental systems. Similarly,
a number of variables must be considered in extrapolating
in vivo data from non-human systems to estimate
antihyperplastic effectiveness of antibodies in humans.
30　In particular, it is important to consider any
differences in the nature and severity of a blood vessel
injury between experimental and clinical systems t best
utilize model data in determining actual treatment
protocols for humans. Likewise, interspecies differences
35　in vascular anatomy and hist l gy, and intrinsic
differences in the hyperplastic processes trigger d by
different kinds of vascular injuries, must be weighed

when extrapolating between model and clinical applicati ns. Nevertheless, the assays and methods described herein, including the *in vitro* and *in vivo* studies using non-human and human primate model systems, provide all of the necessary guidance, coupled with known techniques including standard clinical trial procedur , to determine successful treatment protocols for intimal hyperplasia in mammalian patients, including humans.

It is preferred that the antihyperplastically effective amount of antibody or other antag nist significantly inhibit proliferation (e.g., as determined in an *in vitro* mitogenesis assay) and/or migration of vascular smooth muscle cells. A "significant" reducti n is a reduction of mitogenesis or migration of 50% or m re in an *in vitro* assay. While the actual amount will depend in part on such factors as the specificity and binding affinity of a particular antibody, an effective amount can be determined empirically by *in vitro* and *ex vivo* procedures known in the art and disclosed herein. In general, amounts of PDGF antagonist for therapeutic use will be sufficient to provide a concentration in the bloodstream or at the site of action at least equal t that shown to be effective *in vitro* or *ex vivo*. It is preferred, however, to use higher amounts *in vivo*, up to or exceeding an order of magnitude increase. Thus, for example, in model systems anti-PDGF receptor antibody dosage is selected with the goal of providing temporary or persistent, local or systemic levels of antibody in the treated mammal which correspond to antibody concentrations shown to be antihyperplastically effective in suitable *in vitro* tests.

Of particular interest for *in vivo* testing is a baboon vascular injury model disclosed in detail herein. This model has been designed to mimic the injury response that occurs in humans f ll wing vari us types of acute treatments to pen occl ded arteries. The use of balloon angioplasty for the generati n of a vascular lesion

mimics a procedure that is commm nly used to r -establish blood flow in stenosed coronary arteries and which leads to restenosis in 30-40% of treated individuals. This model is therefore particularly well suited for testing the use of anti-PDGF receptor antibodies or other PDGF antagonists, alone or in conjunction with heparin.

Another suitable model for testing the efficacy of PDGF antagonist therapy is a baboon model of car tid endarterectomy. In this model an acute injury is made t the medial area of the artery, which subsequently leads to the development of un intimal lesion (Hanson et al., Hypertension 18:I170-I176, 1991). This model mimics the use of carotid endarterectomy to open carotid arteries in humans that have decreased blood flow due to advanced atherosclerosis. A third model for testing the use of PDGF antagonist therapy is a baboon vascular graft emplacement model. It has been demonstrated that the placement of vascular grafts leads to the generati n of hyperplastic lesions at the site of the graft (Kraiss et al.,J. Clin. Invest. 92:338-348, 1993). These lesi ns have characteristics similar to those of hyperplastic lesions in humans at sites of vascular injuries.

To test the efficacy of PDGF antagonist therapy in humans, various types of analysis can be used. These include monitoring for a loss in mean lumenal diameter (MLD) by angiography at the 3-6 month period foll wing vascular treatment. Alternative methods to monit r efficacy include intravascular ultrasound, B-mode ultrasound and magnetic resonance imaging. Clinical correlates can also be used to monitor for efficacy of therapy. These include a decrease in myocardial infarcts and recurent angina, and the need for repeat r - vascularization.

Antibody dosage levels are calculated from inhibiti n data after determining clearance of antibody fr m the bl od. In general, dosage is selected with the goal of maintaining circulating levels of antibody

sufficient to inhibit greater than 10%, preferably at
least 20-50% of circulating PDGF activity (e.g.,
receptor-ligand binding, PDGF- or serum-stimulated
mitogenesis and/or migration of VSMCs, or an ther
5   biological activity correlated with PDGF recept r
function and/or regulation of an intimal hyperplastic
process). In general, doses will be in the range of
about 0.1 μg to 500 mg or more of antibody per kg of
patient body weight per day, preferably about 20 μg to 20
10   mg/kg/day, more preferably about 1 mg-10 mg/kg/day. As
noted above, the actual dose will depend in part n
antibody affinity and activity. Somewhat higher doses
may be required if two or more antibodies are
administered in combination than if a single antibody is
15   used. To minimize antibody production costs and limit
immuno-intolerance of administered antibodies by the
patient, it is preferred to use high affinity antibodies
having a high specific inhibitory activity, enabling the
use of doses of about 1 mg/kg/day or less. Doses of n n-
20   antibody PDGF antagonists can be determined using similar
criteria.

In humans treated with anti-PDGF recept r
antibody therapy, either alone, or in combination with
heparin, the antibody may be given under a wide range f
25   conditions. The antibody can be given via bolus
injections, both prior to the re-vascularization
procedure as well as multiple times following the
procedure. The antibody may be given as a bolus
injection (intravenous, intramuscular, intraperitoneal or
30   subcutaneous) prior to the procedure (generally within 24
hours before surgery) and a constant infusion following
the procedure (including infusion via implanted pumps).
In many cases it will be preferable to administer daily
doses (including administration by infusion) during a
35   h spital stay, f llowed by less fr quent bolus injections
during a period of outpatient treatment of one to two
weeks or m re. Treatment may be continued for up to six

months after initial injury. The antibody may be given via multiple routes including intravenous, intramuscular or subcutaneous injections. In addition the antibody may be delivered locally to the site of vascular injury using perfusion balloon cathethers, coating onto stents, or placement on gel coated balloons. In the latter cas s it would be expected that the doses of antibody would be substantially less than that required when given systemically. The antibodies may also be delivered by slow-release delivery systems, including such systems incorporated into vascular grafts or stents, or by way of perfusion or double balloon catheters. For inhibiti n of stenosis in vascular grafts, anti-PDGF receptor antibodies may be covalently attached to the graft through their constant regions or incorporated int the graft in slow-release formulations. Pumps and other known delivery systems may also be employed. In any event, administration is designed to provide the desired daily dose (e.g., a five-day bolus of 25 mg/kg to pr vide 5 mg/kg/day). Mode and timing of administration of ther PDGF antagonists can be determined from chemical and physical properties of the specific antagonist and pharmacokinetic data according to accepted principles.

For use within the present invention, anti-PDGF receptor antibodies are formulated into injectable compositions according to conventional procedures and packaged in sterile containers. The antibodies may be combined with a suitable diluent such as sterile saline or sterile water. The antibody compositions may further contain carriers, stabilizers and excipients such as sugars (e.g. mannitol) or albumin. In the alternative, the antibodies may be provided in lyophilized form and reconstituted in a suitable diluent prior to use. These compositions may be packaged in single or multiple dosage form, for exampl in sealed amp ules or vials. Non-peptidic PDGF antagonists may be delivered parenterally or enterally (.g. orally).

In an alternate embodiment f th inventi n, an anti-growth fact r receptor antibody r n n-antibody PDGF antagonist is administered to a mammal coordinately with heparin, in respective unit doses of antibody/antagonist and heparin sufficient to combinatorially inhibit intimal hyperplasia in the vasculature of the mammal. In this context, "coordinate administration" is intended to include concurrent, separate or sequential adminstration of the antibody/antagonist and heparin, wherein both the antibody/antagonist and heparin are administered within a limited, combinatorially effective time period relative to one another. A "combinatorially effective time period" is defined as a maximum intervening time period between administration of the antibody/antagonist and administration of the heparin in which the two agents are combinatorially effective in inhibiting the hyperplasia. The term "combinatorially effective" is in turn defined as producing a measurable inhibition of intimal thickening or lesion formation, or of a hyperplastic process, which exceeds a maximum level of inhibition independently provided by either the antibody or heparin administered alone, under otherwise comparable conditi ns and dose.

As used herein, the term "heparin" refers to any member of a family of structurally complex, sulphated glycosaminoglycans generally characterized by a structur of repeating glucosamine and glucuronic acid sugar residues (Casu, Adv. Carbohyd. Chem. and Biochem. 47: 578-583, 1985). The most widely known heparin is "unfractionated" or "commercial" heparin prepared from bovine lung or porcine gut, which encompasses a heterogeneous mixture of heparin molecules ranging from approximately 8,000 to 20,000 daltons molecular weight (Wolinsky et al., J. Am. Coll. Cardiol. 15: 475-481, 1990). However, th term heparin also encompasses a br ad range f more homogeneous heparin preparations, as well as h parin-lik m lecules, including heparan

sulfates. Am ng these particular heparin xamples, more specific heparin subtypes are also kn wn. For example, heparan sulfate moieties produced by endothelial cells (Castellot et al., *J. Cell. Biol.* 90: 372-379, 1981) and smooth muscle cells (Fritze et al., *J. Cell. Biol.* 100: 1041-1049, 1985) have been isolated which are reportedly up to 40 times more active than unfractionated heparin for inhibiting proliferation of smooth muscle cells. In addition, among the naturally occurring heparin size variants, fractionated heparin species that exhibit predominantly either anticoagulant or antiproliferative activity have been isolated (Wolinsky et al., *J. Am. Coll. Cardiol.* 15: 475-481, 1990). The latter activity tends to be present in the low molecular weight heparin species, such as heparins in the range of penta- to decasaccharides, which have been reported to also provide greater bioavailability and a longer half-life (*Id.*, Bacher et al., *Thrombosis Res.* 70: 295-306, 1993), and may therefore be particularly useful within specific embodiments of the invention. Also included within the definition of heparin for the purposes of describing the invention are synthetic heparins and heparin derivatives, a variety of which have been produced using conventional chemical synthetic, modifying and degradative techniques (see for example, Roden, L. *The Biochemistry of Glycoproteins and Proteoglycans* (Lennarz, W.J., ed.) pp 267-371, Plenum Publishing Corp., New York, 1980, incorporated herein by reference). The term "low molecular weight heparin having reduced antithrombotic activity" is used to indicate low molecular weight forms having reduced antithrombotic activity (as determined by standard assays) compared to unfractionated heparin.

To determine combinatorially effective doses of antibody/antagonist and heparin, and/or to evaluate combinatorially effective time periods for separately or sequ ntially administering a PDGF antagonist and heparin, the same general methods described above for assaying

antihyp rplastic activity f anti-PDGF receptor antibodies, and for extrapolating between experimental and clinical applications, are used. These methods include mitogenesis and migration assays using VSMC cell cultures or vascular tissue explants, as well as a variety of *in vivo* assays which measure the incidence r degree of intimal hyperplasia in a living subject, among others. From these methods, it is either demonstrated or expected that the level of combinatorial inhibition achieved by coordinately administering antibody or oth r antagonist in combination with heparin varies depending on the respective types and dosages of antagonist and heparin used, on the timing and mode of administration f the antagonist and heparin, and upon other experimentally and clinically relevant variables, such as the type of cells or tissues treated, or the nature and severity of a blood vessel injury. By adjusting the coordinate administration regimen (eg. heparin and antagonist types, dosages, and modes or timing of administration) within the methods of the invention, combinatorial inhibition f intimal hyperplasia can be optimized to facilitate a broad range of specific applications of the invention. For example, different antibody and heparin types and dosages may be desired for different clinical applications. For patients at high risk of thrombosis-related complications, anticoagulant forms of heparin may be clinically desirable. Other patients may be particularly vulnerable to bleeding complications related to the use of anticoagulant forms of heparin, in which case a low molecular weight heparin having reduced antithrombotic activity may be indicated. These and other clinical considerations will be evident to those skilled in the art.

To accomodate the choice of a particular heparin type r dose, the methods of the invention allow for co-variati n of the form, timing or dose of antibody of other antag nist to be coordinat ly administered, such

that the administrati n r gimen f r the antibody r other antagonist can be coordinately adjusted to maintain a high level of combinatorial inhibition. In other circumstances, the form or dosage of antagonist, r the timing or mode of administration of antagonist may be imposed by extrinsic circumstances, in which case th heparin administration regimen may need t be coordinately adjusted. For example, in circumstances where a prolonged antibody treatment regimen is desir d, lower antibody doses, or less immunogenic antibody forms (e.g., mouse/human chimeric antibodies) may be us d to optimize results. In such cases, a coordinate adjustment can be made with respect to the type, dose or timing of administered heparin to achieve a strong, combinat rial antihyperplastic effect.

Using the coordinate administration methods of the invention, dosages of antibody or other antagonist and heparin in particular may be coordinately varied across a broad range while maintaining a high level of combinatorial inhibition of intimal hyperplasia. This feature of the invention is especially useful for accomodating clinical applications where a low dose of one or the other antihyperplastic agent (i.e. the antagonist or heparin) is desired, such as in cases where dose-limiting toxicities, allergies or other complications are present. Within the methods f the invention, coordinately administered anti-PDGF recept r antibodies and heparin have been found to be combinatorially effective in antibody:heparin dose ratios (i.e. ratio of unit antibody dose to unit heparin dose, by weight) ranging between .001:1 to 1,000:1, and broader. In other words, a unit dose of antibody as low as 1/1,000 of a dose of coordinately administered heparin yields a combinatorially inhibitory effect, while antibody d ses 1,000 f ld gr ater than a coordinately administered heparin dos als yields a combinatorial effect. This g nerally inverse-proportional co-

variability of antibody and heparin doses pr vides extreme flexibility f r implementing alternative, coordinate administration regimens using the two antihyperplastic agents. At the same time, less extreme co-variation of antibody and heparin doses, embodied in antibody:heparin dose ratios between .01:1 and 100:1, and between .05:1 and 20:1, also been shown t be combinatorially effective, and are preferably selected in circumstances where moderate to extremely low doses of both the antibody and heparin are clinically desired.

In general, doses of antibody to be coordinately administered with heparin for treating intimal hyperplasia in mammals will be in the range f between approximately 0.1 μg-100 mg of antibody per kilogram of body weight of the mammal per day. Preferably, doses will be between approximately 50 μg-20 mg of antibody per kilogram per day, and more preferably less than 1 mg/kg/day, to conserve expensive antibody stocks and limit side effects while yielding satisfactory levels of inhibition. Generally, doses of heparin will be between approximately 1 μg-100 mg/kg/day. Preferably, heparin doses will be between 20 μg-10 mg/kg/day, and more preferably less than about 1 mg/kg/day. M re specifically, coordinately administered doses of antibody and heparin of between approximately 0.5 μg-10 mg/kg/day, and between approximately 1 μg-10 mg/kg/day, respectively, yield strong combinatorially effective results at relatively low doses of both antihyperplastic agents. Where even lower doses of antibody and heparin are desired, coordinately administerd amounts of antibody and heparin of between approximately 5 μg-2 mg/kg/day, and between approximately 50 μg-1 mg/kg/day, respectively, are preferred. Those skilled in the art will recognize that actual doses will be determined with c nsiderati n of specific circumstances, including patient parameters and the characteristics of the antibodie(s) (e.g., specificity, sp cific activity,

circulating half-life) and heparin (e.g., antithrombotic activity) administ red.

Anti-PDGF receptor antibodies and heparin are preferably administered parenterally, such as by bolus injection or infusion (intravenous, intramuscular, intraperitoneal or subcutaneous) prior to surgery (generally within 24 hours before surgery) and opti nally continuing after surgery at intervals of from s veral hours to several days over the course of one to two weeks or more. Within one embodiment, the antibody is administered as a bolus injection or infusion on the first day of treatment in an amount suficient to pr vide a minimum circulating level of antibody throughout the intitial, three-day treatment period of between approximately 20 µg and 1 mg/kg body weight. In this regard, it is preferred to use antibodies having a circulating half-life of at least 12 hours, preferably at least 4 days, more preferably up to 14-21 days. Chimeric and humanized antibodies are expected to have circulatory half-lives of up to four and up to 14-21 days, respectively. In many cases it will be preferable t administer daily doses during a hospital stay, foll wed by less frequent bolus injections during a period of outpatient treatment. The antibodies and heparin may also be delivered by slow-release delivery systems, including such systems incorporated into vascular grafts or stents, or by way of perfusion or double balloon catheters. Pumps and other known delivery systems may also be employed for continuous infusion. Dosing regimens may be varied to provide the desired circulating levels of antibody and heparin based on the pharmacokinetics of these agents. Thus, doses will be calculated so that the desired circulating levels of therapeutic agents are maintained. Daily doses referred to above may be administered as larger, less frequent bolus administrati ns t pr vid the recited dose

averag d over the term of administrati n. Non-peptidic
PDGF antagonists may be administered enterally.

For use within the present invention, anti-PDGF
receptor antibodies, other PDGF antagonists, and heparin
5   are combined or separately formulated into compositions
suitable   for   parenteral   (e.g.,   intravascular,
perivascular   or   transdermal),   oral   or   rectal
administration according to conventional procedures and
packaged in sterile containers. The antagonists and
10   heparin may be jointly or separately combined with a
suitable diluent such as sterile saline or sterile water.
The   antagonist,   heparin   and   antagonsit/heparin
combination compositions may further contain carriers,
stabilizers and excipients such as sugars (e.g. mannitol)
15   or albumin. In the alternative, the antagonists and
heparin may be provided in lyophilized or other stabl ,
dry form and reconstituted in a suitable diluent (which
may be included with the antagonist and heparin) prior to
use. These compositions may be packaged in single or
20   multiple dosage form, for example in sealed ampoules r
vials. For alternative modes of administration, such as
for endovascular administration to inhibit stenosis in
vascular grafts, anti-PDGF receptor antibodies and/or
heparin may be covalently attached to the graft thr ugh
25   their constant regions or incorporated into the graft in
slow-release formulations.

The following examples are offered by way of
illustration, not by way of limitation.

30   Examples
Example 1 discloses the preparation of
hybridomas producing monoclonal antibodies to the PDGF
receptor alpha and beta polypeptides. Examples 2, 3 and
4 disclose the identification and characterization of
35   anti-PDGF-beta rec ptor mon clonal antibodies. Example 5
discl ses the identificati n and characterization of
anti-PDGF-alpha receptor monoclonal antibodies. Example

6 discl ses the determinati n f the binding specificiti s of certain representative monoclonal antibodies. Example 7 demonstrates the inhibition f PDGF mitogenic activity on human dermal fibroblasts using anti-PDGF receptor monoclonal antibodies. Example 8 demonstrates the inhibition of PDGF mitogenic activity n baboon smooth muscle cells using anti-PDGF receptor monoclonal antibodies. Examples 9 and 10 disclose the use of anti-PDGF receptor monoclonal antibodies t inhibit baboon serum mitogenic activity. Example 11 demonstrates the inhibition of baboon aortic smooth muscle cell migration by anti-PDGF receptor monoclonal antibodies. Example 12 demonstrates the ability of anti-PDGF receptor MAbs to inhibit PDGF activity up to eight hours after the ligand has bound to receptors. Example 13 discloses the displacement of receptor-bound PDGF from human osteosarcoma cells by anti-PDGF receptor MAbs. Example 14 demonstrates the inhibition of PDGF and babo n serum mitogenic activity on vascular smooth muscle cells using anti-PDGF receptor monoclonal antibodies administered alone or coordinately administered with heparin. Example 15 discloses the use of heparin, alone or coordinately administered with anti-PDGF recept r monoclonal antibodies, to inhibit serum mitogenic activity on baboon vascular smooth muscle cells. Example 16 discloses further studies demonstrating inhibiti n f serum mitogenic activity on baboon smooth muscle cells using anti-PDGF receptor monoclonal antibodies coordinately administered with heparin. Examples 17 and 18 disclose studies comparing the antimitotic activities of parent murine and mouse/human chimeric anti-PDGF-alpha and beta receptor antibodies coordinately administered with heparin. Example 19 further describes the inhibitory activity of coordinately administered heparin and anti-PDGF r cept r antibodies against serum mitogenic activity. Example 20 demonstrates th inhibition of sm oth muscle cell outmigration from baboon aortic

explants by anti-PDGF recept r mon clonal antibodies ccordinately administered with h parin. Examples 21-23 disclose binding studies of PDGF and anti-PDGF receptor antibodies, in combination and in the presenc and absence of heparin, to determine potential binding or activity interactions between PDGF and heparin, and the anti-PDGF receptor antibodies and heparin. Exampl 24 describes studies to monitor circulating levels of anti-PDGF receptor antibodies following continuous infusi n f the antibodies into a baboon, and to measure baboon antibodies generated against the anti-PDGF receptor antibodies. Example 25 discloses studies to determine the *in vivo* half-life of a chimeric anti-PDGF recept r antibody in a primate model. Example 26 describes a sequential arterial injury model in baboons for testing antihyperplastic agents and treatments following vascular injury. Example 27 describes a baboon model useful in characterizing the role of anti-PDGF receptor antibodies and heparin in inhibiting intimal hyperplasia in primates following acute vascular injury.

Recombinant PDGF AA and BB were produced in yeast essentially as disclosed in U.S. Patents Nos. 4,889,919; 4,845,075 and 5,037,743, which are incorporated herein by reference in their entirety, and purified to homogeneity from concentrated cell culture media by a combination of cation exchange chromatography, reverse-phase chromatography, gel filtration and $(NH_4)_2SO_4$ fractionation. PDGF AB was prepared from outdated human platelets as disclosed by Hart et al., (Biochemistry 29: 166-172, 1991), which is incorporated herein by reference. PDGF-AA, AB and BB were labeled with ^{125}I by use of Iodobeads™ (Pierce Chemical Co., Rockford, IL) as previously described (Hart et al. ibid.) A mutant form of B-chain termed BB_{tyr}, which has a tyrosine residue at position 23 of the mature coding sequence in place of phenylalanine, was used for iodination of PDGF-BB. Rabbit anti-mouse IgG and MAb

163.31 were similarly radi labeled with ^{125}I using Iodobeads™.

Fusion proteins comprising a human IgG heavy or light-chain joined to the extracellular domain of either the PDGF-alpha receptor or PDGF-beta receptor were prepared essentially as disclosed in U.S. Patent N . 5,155,027; U.S. Patent Application Serial No. 07/634,510 and EP 325,224, which are incorporated herein by reference in their entirety. In one case mouse myel ma cells were transfected with cDNAs for both heavy-chain and light-chain/PDGF receptor extracellular domain fusi n proteins. These cells secrete into their culture media a molecule which is analogous to human IgG in that it is composed of two light-chain and 2 heavy-chain fusi n proteins. This compound is designated as tetrameric IgG/PDGFr. In another case a cDNA for light-chain/PDGF receptor extracellular domain fusion protein was transfected into the cells alone. These cells secrete monomeric light-chain fusion proteins into their culture media, designated as monomeric IgG/PDGFr. The alpha- and beta-receptor fusion proteins were designated IgG/PDGFr-alpha (tetrameric) and IgG/PDGFr-beta (monomeric and tetrameric), respectively. The fusion proteins w re purified by either immunoaffinity purification using anti-PDGF receptor monoclonal antibodies, or by pr tein A-Sepharose™ chromatography.

Example 1
Preparation of PDGF Receptor Monoclonal Antibodies

Fusion proteins comprising an IgG constant region joined to the extracellular domain of either the PDGF-alpha receptor (PDGFr-alpha) or the PDGF-beta receptor (PDGFr-beta) were prepared essentially as disclosed in U.S. Patent N . 5,155,027, which is incorp rated herein by referenc in its entirety. The alpha and beta receptor fusions were designated

IgG/PDGFr-alpha and IgG/PDGFr-beta, resp ctively. The
m nomeric IgG/PDGFr-beta was expr ssed as a fusi n of a
human kappa light chain constant region and the PDGF-beta
receptor extracellular domain. The tetrameric IgG/PDGFr-
5   beta was prepared by coexpression of the monomeric
construct with a human Ig heavy chain constant region
plus hinge sequence fused to the extracellular domain.
Alpha receptor fusions were prepared by similar means.

Eight-week-old Balb/c mice were immunized with
10   either purified monomeric or tetrameric IgG/PDGFr-beta or
purified tetrameric IgG/PDGFr-alpha. Mice were given
intraperitoneal (ip) injections of approximately 10 μg f
purified IgG/PDGFr mixed with complete Freund's adjuvant.
At approximately 2 week intervals the mice received
15   additional ip injections of IgG/PDGFr-beta or IgG/PDGFr-
alpha mixed with incomplete Freund's adjuvant.

Hybridomas were prepared from the immunized
mice essentially as disclosed in U.S. Patent N .
5,094,941, which is incorporated herein by reference in
20   its entirety. Briefly, spleen cells were isolated from
the mice and washed. Contaminating red blood cells w re
removed by lysing with distilled water, and the spleen
cells were washed. Any remaining contaminating tissue
material was removed by centrifugation.

25   The NS-1 mouse myeloma cell line (ATCC TIB 18)
was used for the fusions. To optimize fusion efficiency,
cells were assayed for fusion efficiency, and a clon
with a high fusion efficiency was selected. The NS-1
cells were grown in NS-1 media (Table 1) at 37°C, 7% CO_2.

30   Thymocytes obtained from baby mice were used as
a feeder layer to condition the culture medium for the
cell fusions. Thymus glands were obtained from three- to
four-week old Balb/c mice, and thymocytes were isolated
as disclosed in U.S. Patent No. 5,094,941.

35   NS-1 cells were added t the prepared immunized
mouse spleen cells and fusion was carried out essentially
as disclosed in U.S. Patent N . 5,094,941. The cells

were cultured in NS-1 medium c ntaining 1 x HAT (Tabl 1)
and 2.5×10^6 thymocytes p r ml. The hybridomas were
tested between days 9 and 14 for the production of
specific antibodies. Cell fusions were designated by
5 number (e.g., 162, 163).

TABLE 1

NS-1 Medium

For a 500 ml solution:

10 5 ml   10 mM MEM non-essential amino acids (GIBCO BRL,
            Gaithersburg, MD)

    5 ml   100 mM sodium pyruvate (Irvine, Santa Ana, CA)

    5 ml   200 mM L-glutamine (GIBCO BRL)

    5 ml   100x Penicillin/Streptomycin/Neomycin (GIBCO BRL)

15 75 ml   inactivated fetal calf serum (BioCell, Carson, CA)

    1 gm   NaHCO$_3$

        Add RPMI 1640 (GIBCO BRL) to a total volume of
            500 ml. Sterilize by filtration through a 0.22
            lm filter.

20 Table 1, continued

100x HT Stock

38.5 mg   thymidine

136.10 mg hypoxanthine

        Dissolve the thymidine and hypoxanthine in
25   distilled H$_2$O and bring volume up to 100 ml.
            Warm the solution to 60-70°C to dissolve the
            solids. After the solids have dissolved,
            readjust the volume to 100 ml. Sterilize by
            filtration through a 0.22 μm filter. Store
30   frozen at -20°C.

Table 1, continued

1000x A Stock

17.6 ng aminopterin

    Add sterile distilled water to the aminopterin
5   and bring the volume to 50 ml. Add 1 N NaOH
    drop-wise until the aminopterin dissolves.
    Bring the final volume to 100 ml with distilled
    H_2O. Sterilize by filtration through a 0.22 lm
    filter. Store frozen at -20°C.

10

50x HAT

50 ml 100x HT

5 ml   1000x A stock

45 ml   distilled H_2O

15   Sterilize the solution by filtration through a
    0.22 lm filter. Store frozen at -20°C.

ELISA A Buffer

0.1 M Na_2HCO_3, pH 9.6

20   0.02% NaN_3

ELISA B Buffer

This buffer may be made with 1% or 2% bovine serum
albumin (BSA, available from Sigma Chemical Co., St.

25 Louis, MO)

5 or 10 µg BSA (for 1% or 2% BSA, respectively)

250 µl   Tween 20 (Sigma)

100 mg NaN_3

    Add phosphate-buffered saline pH 7.2 (PBS,

30   Sigma) to a final volume of 500 ml.
    Alternatively, the buffer may be made up as 1%
    or 2% BSA in ELISA C Buffer.

ELISA C Buffer

35   500 µl   Tween 20 (Sigma)

200 mg NaN_3

    Add PBS t a final volume of 1 liter.

Table 1. continued

Reaction Buffer

10 ml	0.1 M Na-Citrate, pH 5.0
5 mg	o-phenylenediamine Dihydrochloride (Sigma)
5 µl	H_2O_2 (Sigma)

Extraction Buffer

100 ml	PBS
1.0 ml	Nonidet P-40 (NP-40) detergent (Sigma) (1%

final concentration)

Binding Media

500 ml	Ham's F-12 (GIBCO BRL)
12 ml	1 M Hepes pH 7.4
5 ml	100x Penicillin/Streptomycin/Neomycin (GIBCO BRL)
1 gm	rabbit serum albumin (Sigma)

Mito Media

For a 500 ml solution:

250 ml	DMEM (GIBCO BRL)
250 ml	Ham's F-12 (GIBCO BRL)
0.25 ml	10 mg/ml stock of insulin (GIBCO BRL) to give a final concentration of 5 µg/ml
1 ml	10 mg/ml stock of transferrin (Collaborative Research, Bedford, MA) to give a final concentration of 20 µg/ml
2 ml	4 µg/ml stock of selenium (Aldrich Chemical, Milwaukee, WI) to give a final concentration of 5 nM
5 ml	10% stock solution of bovine serum albumin (GIBCO BRL) to give a final concentration of 0.1%.

36

Example 2
Identification and Characterization of Hybridomas
Producing Antibodies to the PDGF Beta Receptor

5   Hybridomas from cell fusion 162 were tested for
the production of antibodies to the PDGF-beta recept r.
Assays used for identification of positive hybridomas
included enzyme linked immunosorbent assays (ELISA),
inhibition of ^{125}I-PDGF-BB binding to IgG/PDGFr-beta, and
10   inhibition of ^{125}I-PDGF-BB binding to human dermal
fibroblasts.
       The ELISA assays were carried out in 96-well
microtiter plates which had been coated with monomeric
IgG/PDGFr-beta. To coat the wells, IgG/PDGFr-beta was
15   diluted to 200 ng/ml in ELISA A buffer (Table 1), and 100
µl of the solution was added to each well. The plates
were incubated at 37°C for 2 hours. After incubation, the
plates were washed with ELISA C buffer (Table 1). The
plates were then incubated with 150 µl/well of ELISA B
20   buffer (Table 1) at 37°C to block nonspecific binding
sites. The buffer was removed, and the wells were washed
with ELISA C buffer.
       The test hybridoma supernatants were pooled in
groups of two, and 100 µl of the pooled samples was added
25   to each of the microtiter wells. The plates w re
incubated for 1 hour at 37°C. The plates were washed with
ELISA C buffer, then incubated for 1.5 hours at 37°C with
biotin-conjugated rabbit anti-mouse IgG (Vector Labs,
Burlingame, CA). The wells were washed with ELISA C
30   buffer, then incubated for 30 minutes at 37°C with 100
µl/well of strepavidin-horseradish peroxidase (Amersham
International, Amersham, U.K.). The wells were washed
again with ELISA C buffer, then incubated with reaction
buffer (Table 1). The reaction was stopped by th
35   addition f 1 N H_2SO_4, and the plates were read in a
Dynatech ELISA plate reader (Dynatech Lab ratori s, Inc.
Alexandria, VA) using a filter t monitor absorbance at

49 nm. Those wells with A_{490} r adings greater than 0.2 were taken as positives. The positive candidates w re re-assayed by ELISA as described above to determine the individual culture wells that contained the hybridoma cells producing antibody to IgG/PDGFr-beta.

Hybridomas from cell fusion 162 were als screened for inhibition of ^{125}I-PDGF-BB binding to IgG/PDGFr-beta. Goat anti-human IgG (Cappel Labs, Malvern, PA) was diluted with ELISA A buffer to a final concentration of 2 $\mu g/ml$. This mixture was then added t 96-well microtiter plates, 100 $\mu l/well$, and the plates were incubated for 1.5 hours at 37°C. The wells were washed with ELISA C buffer, then incubated with 200 μl per well of ELISA B buffer to block nonspecific binding sites. The plates were washed with ELISA C buffer, then incubated for 1.5 hours with tetrameric IgG/PDGFr-beta, and diluted in ELISA B buffer to a final concentration of 25 ng/ml. The wells were washed with ELISA C buffer to remove unbound IgG/PDGFr-beta.

Hybridoma supernatants were pooled in groups of two, and 100 μl of the pooled samples was added to each of the microtiter wells. The wells were incubated f r 1 hour at 37°C. To each well was then added 50 μl of ^{125}I-PDGF-BB (approximately 50,000 cpm per well). After a 1 hour incubation at 37°C the wells were washed three times with binding media (Table 1). 100 μl of 0.1M NaCitrate, pH 2.5, was added to the wells for 5 minutes at room temperature, the solution was harvested and transfered to 12x75 mm tubes, and the tubes were counted in a gamma counter to determine the level of ^{125}I-PDGF-BB binding. Antibodies which bound to IgG/PDGFr-beta and blocked ^{125}I-PDGF-BB binding were detected by a decrease in the level of ^{125}I-PDGF-BB bound, as compared to culture media alone.

P ols f media that w re determined t be positive for IgG/PDGFr·beta neutralizing antibody were rescr ened using an assay f rmat similar to that

described above t identify the individual wells which contained hybridomas producing the neutralizing antibody.

Media samples from culture wells that were positive either by ELISA cr by inhibition of ^{125}I-PDGF-BB

5 binding were subsequently assayed in a down-regulation assay format (Hart et al., _J. Biol. Chem._ _262_: 10780-10785, 1987) for the ability to recognize PDGF-beta receptor on human dermal fibroblasts. The binding of PDGF-BB to the PDGF-beta receptor at 37°C leads to the

10 internalization of the receptors from the cell surface and a subsequent decrease in the number of cell-surface receptors, a phenomenon refered to as down-regulati n. The fibroblasts were plated into 96-well culture dishes at 10,000 cells per well and maintained in culture media

15 for 1-2 days prior to use. To one set of wells was added PDGF-BB at a final concentration of 100 ng/ml on the cells. The cells were incubated for 1.5 hours at 37°C. The culture media was removed from the cells, and the cells were washed with phosphate buffered saline (PBS).

20 Test culture media from the hybridoma cells was then added to duplicate wells of cells that had either received the PDGF-BB treatment or cells that had been left untreated. The cells were subsequently incubated for 2 hours at 4°C, then washed with PBS. 100 μl/well of

25 ^{125}I-rabbit anti-mouse IgG (100,000 cpm/well) was added to the wells, and the cells were incubated f r an additional 1.5 hours at 4°C. The cells were washed with PBS, then incubated for 5 minutes at room temperature with 100 μl/well of extraction buffer (Table 1). The

30 extracts were harvested, transfered to 12x75 mm tubes and counted in a gamma counter to determine the level of ^{125}I-rabbit anti-mouse IgG binding. If there is antibody in the hybridoma culture supernatants capable of recognizing cell-surface PDGF-beta receptor, then there

35 w uld be a decrease in the l vel of ^{125}I-rabbit anti-m use IgG binding to those cells that were treated with PDGF-BB to down-regulat the r cept rs.

Several hybrid mas were id ntified fr m fusi n 162 as making antibody t the PDGF-beta r cept r. The hybridomas identified were twice cloned by limiting dilution to obtain individual clones making monoclonal antibody. The clones were screened for antibody production by the assays described above. One hybridoma, named 162.62, was selected for further characterization.

Example 3
Identification and Characterization of Hybridomas
Producing Anti-PDGF Beta Receptor Antibodies

Hybridomas from cell fusion 163 were tested f r the production of antibodies to the PDGF-beta recept r by a combination ELISA/PDGF binding competition assay. These assays were carried out in 96-well microtiter plates. The plates were initially coated with goat anti-human IgG, 2 μg/ml in ELISA A buffer, for 2 hours at 37°C. The plates were washed with ELISA C buffer, then incubated for 1 1/2 hours at 37°C with ELISA B buff r t block nonspecific binding sites. The plates were washed with ELISA C buffer, then either used immediately or left for 1-4 days at 4°C until use. At the time of the assay the plates were washed once with ELISA C buffer, then incubated for 1 1/2 hours at 37°C with tetrameric IgG/PDGFr-beta diluted to 25 ng/ml in binding medium. The plates were then washed with ELISA C buffer to rem ve unbound IgG/PDGFr-beta.

Hybridoma supernatants were pooled in groups of two wells, and 100 μl of the pooled samples was added t each of the microtiter wells. The plates were incubated for 1 hour at 37°C, then washed with binding medium. To the wells was added horseradish peroxidase-conjugated g at anti-mouse IgG (Tago, Burlingame, CA) diluted 1:1000 with binding medium. The wells were incubat d f r 1 hour at 37°C, th n washed with binding medium t remove unbound

HRP-conjugated g at anti-m use IgG. ^{125}I-PDGF-BB, aproximately 26,000 cpm/w 11, was then add d t the wells for an additional 1 hour at 37°C. The wells were washed with binding medium, then incubated with reaction buffer for development of the ELISA. The reaction was st pped by the addition of 100 μl/well of 1 N H_2SO_4, and the plates were read in a Dynatech ELISA plate reader using a filter to monitor the absorbance at 490 nm.

The contents of the wells were then transfered to 12x75 mm test tubes, and the samples were counted in a gamma counter to measure the level of ^{125}I-PDGF-BB binding.

The above-described assay identified hybrid ma cultures producing antibody to IgG/PDGFr-beta by ELISA, as well as by the ability to block the binding of ^{125}I-PDGF-BB to tetrameric IgG/PDGFr-beta. Those pooled samples that were positive were subsequently reassayed using the same protocol as described above to determine the individual culture wells that contained the hybridoma cells producing antibody to IgG/PDGFr-beta.

Individual wells found to be positive for binding to IgG/PDGFr-beta were subseqently assayed for the ability to inhibit ^{125}I-PDGF-BB binding to human dermal fibroblasts. Human dermal fibroblasts were plated into 24-well culture dishes at approximately 20,000 cells per well. The culture media was removed from the c lls, and hybridoma test culture media, 0.5 ml per well, was added to duplicate wells. As a negative control, NS-1 medium alone as added to one set of wells. To a second set of wells was added PDGF-BB at a final concentration of 20 ng/ml in NS-1 medium to determine non-specific binding of ^{125}I-PDGF-BB. The cells were incubated for 1 hour at 4°C, then ^{125}I-PDGF-BB, 100 μl/well (approximately 26,000 cpm), was added to each well. The cells were incubat d f r an additional 1 hour at 4°C, wash d with PBS, then incubated with extraction buff r. The extracts were harvested t 12x75 mm tub s and count d in a gamma

counter. Test sampl s that caused a decrease in ^{125}I-PDGF-BB binding as c mpared t the NS-1 medium sample were assayed as positive for the ability to inhibit PDGF-BB binding to native PDGF-beta receptor on monolayers f human dermal fibroblasts.

Several hybridomas were identified from fusion 163 to be making antibody to the PDGF-beta receptor. Th hybridomas identified were twice cloned by limiting dilution to obtain individual clones making monocl nal antibody. The clones were screened for antibody production by the assays described above. One hybridoma, named 163.31, was selected for further characterizati n.

Example 4
Characterizaton of Anti-PDGF Beta Receptor MAbs 162.62 and 163.31.

MAbs 162.62 and 163.31 (produced from hybridoma clones 162.62 and 163.31, respectively) were compared for the ability to block the binding of ^{125}I-PDGF-BB to either tetrameric IgG/PDGFr-beta or to PDGF-beta recept r on human dermal fibroblasts. Inhibition of ^{125}I-PDGF-BB binding to IgG/PDGFr-beta was tested essentially as described above for the intital screening of fusion 163. Instead of adding conditioned culture media, known amounts of antibody diluted in NS-1 medium were added simultaneously with ^{125}I-PDGF-BB to the IgG/PDGFr-beta coated wells. NS-1 medium alone was used as a negative control. The addition of PDGF-BB, 500 ng/ml, t NS-1 medium was used to determine the level of nonspecific binding by ^{125}I-PDGF-BB. The wells were incubated at 4°C for 2 1/2 hours, then washed with PBS. 100 μl of 0.1M citrate pH 2.5 was added to each well to remove the bound ^{125}I-PDGF-BB, the samples were transferred to 12x75 mm tubes, and the tubes were then counted in a gamma counter.

T assay binding t human dermal fibroblasts, the fibr blasts were plated at approximately 20,000

cells/w ll in 24-well culture dishes. Th c lls were used for assay 2-7 days after plating. The antibodies were diluted in binding media to the concentrations shown in Table 2, then mixed with ^{125}I-PDGF-BB, and 0.5 ml

5 aliquots were added to duplicate wells of fibroblasts. Binding media alone was used as the negative control, and the addition of 500 ng/ml of PDGF-BB was used to determine nonspecific binding for ^{125}I-PDGF-BB. The cells were incubated for 2 1/2 hours at 4°C, then washed

10 with binding media to remove unbound ligand. The c lls were then incubated with extraction buffer, and the extracts were harvested and counted in a gamma counter.

The results of the binding studies are shown in Table 2. The data are presented as specific cpm bound

15 for ^{125}I-PDGF-BB. Nonspecific binding, determined by the addition of 500 mg/ml of unlabeled PDGF-BB, was 260 cpm for the IgG/PDGFr-beta wells and 105 cpm for the human dermal fibroblasts, and has been subtracted from the data presented. %CB = Percent control binding.

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Table 2

MAb Inhibition of 125I-PDGF-BB Binding to IgG/PDGFr-beta and to Human Dermal Fibroblasts

MAb	Conc. (μg/ml)	IgG/PDGFr CPM	% CB	Fibr blasts CPM	% CB
162.62	1.25	3	0	52	16
	0.62	40	1	71	22
	0.31	71	2	96	30
	0.15	91	3	69	21
163.31	1.25	274	9	244	76
	0.62	499	16	372	116
Control		3062	100	322	100

These results demonstrate that both MAbs 162.62 and 163.31 are potent inhibitors of PDGF-BB binding to IgG/PDGFr-beta. In contrast, MAb 162.62 is a more potent inhibitor than MAb 163.31 for PDGF-BB binding to human dermal fibroblasts

MAb 162.62 was also analyzed for the ability to displace 125I-PDGF bound to receptors on monolayers of human dermal fibroblasts. 125I-PDGF-BB was first incubated with monolayers of human dermal fibroblasts in 24-well culture plates. The cells were washed with PBS, then subsequently incubated for 1 hour at 4°C with either MAb 162.62, 5 μg/ml, or binding medium alone. The cells were washed, incubated with extraction buffer, and the extracts were counted in a gamma counter to determine the level f 125I-PDGF-BB binding. To determin nonspecific binding, 500 ng/ml of unlabel d PDGF-BB was added during

the first incubation step. The results, pr sented in Table 3, show that th additi n f MAb 162.62 led t a 47% displacement of p:abound ^{125}I-PDGF-BB. Thus, MAb 162.62 was able to displace receptor-bound PDGF-BB fr m the surface of human dermal fibroblasts.

Table 3
Ability of MAb 162.62 to Displace Receptor-bound ^{125}I-PDGF-BB From Human Dermal Fibroblasts

1st Inc.	2nd Inc.	CPM Bound	BB Removal
^{125}I-BB	Binding Media	581	
^{125}I-BB	MAb 162.62	308	47 %

The subclass for MAbs 162.62 and 163.31 were determined by ELISA using IgG/PDGFr-beta coated wells and subclass specific secondary antibody. MAb 162.62 was found to be an IgG2b isotype while MAb 163.31 was found to be an IgG1 isotype.

Example 5
Identification and Characterization of Hybridomas Producing Anti-PDGF Alpha Receptor Antibodies

Hybridomas from cell fusion 169 were tested f r the production of antibodies to the PDGF-alpha receptor by a combination ELISA/PDGF binding competition assay. These assays were carried out in 96-well microtiter plates. The plates were initially coated with goat anti-human IgG, 2 μg/ml in ELISA A buffer, overnight at 4°C. The plates were washed with ELISA C buffer, then incubated with ELISA B buffer to block nonspecific binding sites. The plates were washed with ELISA C buffer, then incubated overnight at 4°C with tetrameric IgG/PDGFr-alpha diluted t 25 ng/ml in binding m dium.

The plat s were th n wash d with ELISA C buffer t remove unbound IgG/PDGFr.

Hybridoma supernatants were pooled in groups of two, and 75 μl of the pooled samples was added to each of the microtiter wells. The plates were incubated for 1 hour at 37°C, then washed with ELISA C buffer. T the wells was added horseradish peroxidase-conjugated g at anti-mouse IgG (Tago) diluted 1:1000 with binding medium. The wells were incubated for 1 hour at 37°C, then washed with ELISA C buffer to remove unbound antibody. ^{125}I-PDGF-AA, approximately 25,000 cpm/well, was then added to the wells for an additional 1 hour at 37°C. The wells were washed with binding medium, then inubated with reaction buffer for development of the ELISA. The reaction was stopped by the addition of 100 μl/well f 1 N H_2SO_4 and the plates read in a Dynatech ELISA plate reader using a filter to monitor the absorbance at 490 nm.

The contents of the wells were then transfered to 12x75 mm test tubes, and the samples were counted in a gamma counter to measure the level of ^{125}I-PDGF-AA binding.

This assay identified hybridoma cultures producing antibody to IgG/PDGFr-alpha by ELISA and monitored for antibody which was able to block the binding of ^{125}I-PDGF-AA to the tetrameric IgG/PDGFr-alpha. Those pooled samples which were positive in the initial assay were reassayed using the same protocol as described above to determine the individual culture wells that contained the hybridoma cells producing antibody for IgG/PDGFr-alpha. Several wells were identified for the presence of antibody directed against IgG/PDGFr-alpha. Of these, two were selected for further analysis, 169.14 and 169.31. Hybridomas from these wells were cloned twice by limiting dilution t obtain single clon s producing m noclonal antibody against the PDGF-alpha recept r. Th clones were screened using the combination

ELISA/^{125}I-PDGF-AA binding competiti n assay ssentially as described abov .

To verify that MAbs 169.14 and 169.31 recognize native PDGF-alpha receptor on monolayers of mammalian cells, the two antibodies were analyzed for the ability to block ^{125}I-PDGF-AA binding to alpha T-7 cells. Thes cells are canine kidney epithelial cells that d not naturally express PDGF-alpha receptor, but have been transfected with a cDNA coding for the full length PDGF-alpha receptor (U.S. Patent No. 5,371,205o. 5,371,205; PCT Publication WO 90/14425). These cells express approximately 100,000 recombinant receptors per cell. The alpha T-7 cells were cultured in 96-well plates to approximately 95% confluency. The culture medium was removed, and dilutions of MAbs 169.14 and 169.31 were added to the cells. Controls were NS-1 medium, and NS-1 medium containing 500 ng/ml of PDGF-BB to determine the nonspecific binding component for ^{125}I-PDGF-AA. To each well was added 100 µl of the test sample plus 10 µl of ^{125}I-PDGF-AA (approximately 22,000 cpm per well). The cells were incubated with the samples for 2 hours at 4°C, washed with PBS, then extracted with 100 µl/well of extraction buffer. The extracts were harvested and counted in a gamma counter. The results are shown in Table 4. These results demonstrate that these two MAbs recognize membrane-bound PDGF-alpha receptor in mammalian cells in addition to IgG/PDGFr-alpha.

Table 4

Competition for PDGF-alpha Receptor Binding on Alpha T-7 Cells Between Anti-PDGF Alpha Receptor MAbs and ^{125}I-PDGF-AA

MAb Conc. (µg/ml)	MAb 169.14		MAb 169.31	
	CPM	% CB	CPM	% CB
1.5	2	1	8	2
0.75	2	1	15	4
0.37	7	2	18	5
0.18	5	2	15	4

CPM = counts per minute of ^{125}I-PDGF-AA bound in the presence of antibody. The nonspecific value, 28 cpm, which was determined by the addition of 500 ng/ml of PDGF-BB to the wells, has been subtracted from the CPM values given. Control binding was 392 cpm. % CB= percent control binding.

The subclass for MAbs 169.14 and 169.31 was determined by ELISA using IgG/PDGFr-alpha coated wells and subclass-specific secondary antibody. Both MAbs 169.14 and 169.31 were assayed positive for IgG2a isotype.

Example 6

Binding Specificity of Anti-PDGF Receptor MAbs

To demonstrate PDGF-receptor subunit binding specificity, MAbs 162.62, 163.31, 169.14 and 169.31 were analyzed for binding to Clone 8 cells and Alpha 1-10 cells. Clone 8 cells are BHK 570 (ATCC CRL 10314) cells that have been transfected with a gen coding for the full length human PDGF-beta r ceptor (Gr nwald et al.,

Proc. Natl. Acad. Sci. USA 85: 3435-3439, 1988). Th s
cells express approximately 500,000 human PDGF-beta
receptors per cell. The Alpha 1-10 cells are BHK 570
cells that have been transfected with a cDNA coding for
the full length human PDGF-alpha receptor (U.S. Patent
No. 5,371,205; PCT Publication WO 90/14425). These c lls
express approximately 1,000,000 human PDGF-alpha
receptors per cell. To demonstrate binding specificity
for either the PDGF-alpha or beta receptor, cell surface
binding studies using the anti-PDGF receptor MAbs were
done with these two cell lines.

Both the Clone 8 and Alpha 1-10 cells were
cultured in 24-well plates to confluency. PDGF-BB (200
ng/ml) was added to one-half of the cells to stimulate
PDGF receptor down-regulation, and vehicle control (10 mm
acetic acid, 0.25% rabbit serum albumin) was added t the
other half. The cells were incubated for 1-2 hours at 37°
C, then washed with PBS chilled to 4°C. Purified MAbs
162.62, 163.31, 169.14 and 169.31, diluted to 5 μg/ml in
binding medium, were added to triplicate wells of th
PDGF-BB-treated and nontreated control cells. The c lls
were incubated for approximately 2 hours on ice, then
washed with chilled PBS to remove unbound antibody. Th
test wells were then incubated on ice for 30 minutes with
^{125}I-labeled rabbit anti-mouse IgG, diluted in binding
medium to approximately 400,000 cpm/well. The wells were
washed with PBS, then incubated with extraction buffer.
The extracts were harvested and counted in a gamma
counter. The results, shown in Figure 1, demonstrat d
that only MAbs 162.62 and 163.31 bound specifically to
the PDGF-beta receptor, as demonstrated by the
significant decrease in binding to the PDGF-BB treated
Clone 8 cells when compared to untreated controls. The
high level of binding by MAb 169.14 was due t an
elevated level f nonspecific binding by this antibody,
because th re was n significant decrease in ^{125}I-rabbit
anti-m use IgG binding t the PDGF-BB treated cells. In

c ntrast, only MAbs 169.14 and 169.31 showed binding t the PDGF-alpha rec pt r as dem nstrat d by the specific binding to the Alpha 1-10 cells (Figure 2). Due to the ability of the antibodies to bind to cell surface PDGF receptor, these results confirm that these antibodie recogonize extracellular epitopes on the PDGF receptors.

Example 7
Neutralization of PDGF Mitogenic Activity on Human Dermal Fibroblasts

Human dermal fibroblasts were plated at approximately 20,000 cells per well in 24-well culture dishes and grown until quiescent in DMEM (GIBCO BRL) containing 2% fetal calf serum. The cells were stimulated with either PDGF-AA, AB or BB. Standard curves were run with concentrations of 5, 1.25, 0.31 and 0 ng/ml final concentration on the cells. Stock PDGF dilutions were made with 10 mM acetic acid containing 0.25% rabbit serum albumin, and 50 μl of the stock samples, or vehicle alone, was added to the culture wells to give the desired final concentrations. To analyze the ability of MAbs 162.62 and 169.14 to neutralize the mitogenic activity of each of the three PDGF ligands, 5 ng/ml of PDGF was added to wells along with 20 μg/ml (final concentration on the cells) of MAbs 162.62 and 169.14 alone, or 20 μg/ml of a pool of the two antibodies. The cells were incubated with the test samples for approximately 20 hours at 37°C. The media was aspirated, then replaced with 1 ml of DMEM containing 5% fetal calf serum and supplemented with 1 μCi/ml of [^{3}H]thymidine. The cells were incubated for 4 hours at 37°C, washed with PBS, then harvested with trypsin and counted for [^{3}H]thymidine incorporation in a Wallac (Turku, Finland) Betaplate™ liquid scintillation counter. The results, pres nted in Figur 3A, demonstrate that PDGF-AA mitogenic activity was inhibit d by MAb 169.14

as well as by the antibody po 1, but n t by MAb 162.62. Mitog nic activity of PDGF-AB was inhibited appr ximately 80% by MAb 162.62, and greater than 92% by MAb 169.14 or the antibody pool (Figure 3B). In contrast, activity of

5   PDGF-BB was only minimally inhibited by MAb 169.14, but was inhibited approximately 80% by MAb 162.62 and greater than 92% by the antibody pool (Figure 3C).

These results are consistent with the model of PDGF ligand binding which describes that PDGF-AA binds t

10   PDGF-alpha/alpha receptor dimers, PDGF-AB binds to PDGF-alpha/alpha and -alpha/beta receptor dimers and PDGF-BB binds to all three PDGF receptor dimers; -alpha/alpha, -alpha/beta and -beta/beta (reviewed in Hart et al., _J. Invest. Derm._ 94: 535-575, 1990). Thus, if MAb 169.14

15   binds to and inhibits PDGF binding to the alpha recept r, then it would be expected to inhibit essentially 100% of PDGF-AA and AB mitogenic activity, since alpha receptor binding is required for both of these ligands. This model is consistent with the results described above.

20   The binding to and the inhibition of the PDGF-beta receptor by MAb 162.62 would then be expected to limit the amount of PDGF-AB and BB mitogenic to a level that is consistent with PDGF-AA, since AB and BB would only be able to bind to alpha/alpha dimers. Again, this is

25   consistent with the findings of the study descibed above.

In summary, anti-PDGF-receptor MAbs 162.62 and 169.14 are able to inhibit the mitogenic activity of the three forms of PDGF in manners that are consistent with the current hypothesis as to PDGF receptor binding by the

30   three PDGF ligands. Additionally, the use of the two antibodies in conjunction is able to inhibit essentially 100% of the PDGF mitogenic activity on human dermal fibroblasts.

Example 8
Inhibition of PDGF Mitogenic Activity on Baboon Smooth
Muscle Cells

Anti-PDGF receptor MAbs were analyzed for the ability to inhibit the mitogenic activity of PDGF n baboon smooth muscle cells. All mitogenesis assays performed on baboon vascular smooth muscle cells (BVSMCs) were done on primary cultures of cells between passages 3 and 7 in culture. The initial cultures were established from outgrowth of aortic tissue explants. Baboon smooth muscle cells were plated at approximately 30,000 cells per well, in DMEM supplemented with 10% fetal calf serum, into 24-well culture dishes. Two days prior to use the culture media was removed, and 1 ml of Mito Media (Table 1) was added to each well to allow the cells to become quiescent. At the time of the experiment the cells wer stimulated with either PDGF-AA, AB or BB. Standard curves were run for each of the three ligands using final concentrations shown in Figures 4-6. 20X stock solutions were made for each of the PDGF concentrations by diluti n in 10 mM acetic acid containing 0.25% albumin, and 50 μl of PDGF or dilution vehicle alone was added to the culture wells.

For the mitogenesis assays, final PDGF concentrations of 10, 2 and 1.25 ng/ml were used for PDGF-AA, AB and BB, respectively. MAbs 163.31 and 169.31 were added to the PDGF-containing wells at a final concentration of 25 μg/ml. For pools of the two antibodies, the final concentration of antibody on the cells was 25 μg/ml total, or 12.5 μg/ml for each of the MAbs. The cells were incubated between 20-24 hours at 37° C. For the PDGF-AA and AB studies, 50 μl of a 40 μCi/ml solution of [^{3}H]thymidine was added t each well. For the PDGF-BB study the media was aspirated, then replaced with 0.5 ml of DMEM containing 5% fetal calf serum and

supplemented with 2 μCi/ml f [^{3}H]thymidine. The cells were incubated between 2-4 hours at 37°C, washed with PBS, then harvested with trypsin and counted for [^{3}H]thymidine incorporation in a Betaplate™ liquid scintillation

5 counter (Wallac). As shown in Figure 4, PDGF-AA mitogenic activity was 100% inhibited by MAb 169.31 as well as by the antibody pool, but not by MAb 163.31. PDGF-AB mitogenic activity was completely inhibited by both MAbs individually as well as by the antibody pool

10 (Figure 5). It is interesting to note that the level of [^{3}H]thymidine incorporation in the presence of the MAbs was below the level obtained with the addition of vehicle control only. This was similarly seen with MAb 169.31 n the PDGF-AA plate (Figure 4). For the PDGF-BB stimulated

15 cells, MAb 169.31 and MAb 163.31 gave less than 50% inhibition individually, while a pool of the two antibodies was able to inhibit approximately 75% of the PDGF mitogenic activity (Figure 6).

To further demonstrate the inhibitory potency

20 of these antibodies to neutralize the mitogenic activity of PDGF on baboon smooth muscle cells, two anti-PDGF-alpha receptor MAbs, 169.14 and 169.31, were analyzed for the ability to inhibit PDGF-AA mitogenic activity. BVSMCs were plated and treated essentially as described

25 above. To one set of wells were added increasing concentrations of PDGF-AA in order to generate a standard curve of PDGF-AA mitogenic activity (Figure 7A). The PDGF-AA samples ranged from 10 ng/ml down to 0.31 ng/ml. To a second set of wells, a standard dilution of PDGF-AA

30 was added to give a final concentration of 10 ng/ml. Decreasing concentrations of MAbs 169.14 and 169.31 were then added to the wells to monitor the inhibitory p tency for each of the MAbs, as determined by a decrease in the level of [^{3}H]thymidine incorporation (Figure 7B). The

35 findings demonstrat that even at 8 ng/ml of antibody, there was great r than 90% inhibition of a 10 ng/ml solution of PDGF-AA.

Example 9

Inhibition of Baboon Serum Mitogenic Activity on Baboon Smooth Muscle Cells

Anti-PDGF receptor MAbs were analyzed for the ability to inhibit the mitogenic activity of baboon serum on baboon smooth muscle cells. BVSMCs were plated at approximately 30,000 cells per well, in DMEM supplemented with 10% fetal calf serum, into 24-well culture dishes. Three days prior to use the culture media was rem ved, and 1 ml of Mito Media (Table 1) was added to each well to allow the cells to become quiescent. At the time f the experiment the cells were stimulated with varying amounts of baboon serum.

A standard curve was generated for the serum sample. 20X stock solutions were made for each of the serum concentrations, and 50 μl of the serum dilution or dilution vehicle, PBS, was added to the culture wells to give final serum concentrations on the cells ranging fr m 2.5% down to 0.15%. MAbs 169.31 and 163.31 were analyzed for the ability to inhibit baboon serum mit genic activity. A final serum concentration of 2.5% was used for the antibody inhibition studies. MAbs 169.31 and 163.31 were added to the serum-containing wells at a final concentration of 25 μg/ml. For pools of the two antibodies, the final concentration of antibody on the cells was 25 μg/ml total, or 12.5 μg/ml for each of the MAbs. The cells were incubated with the serum samples for approximately 20 hours at 37°C. At that time the media was aspirated from the cells, then replaced with 0.5 ml of DMEM containing 5% fetal calf serum and supplemented with 2 μCi/ml of [³H]thymidine. The cells w re incubat d f r appr ximately 3 hours at 37°C, washed with PBS, then harvested with trypsin and counted for [³H]thymidine incorporation in a Betaplate™ liquid

scintillation counter (Wallac). The results, pr sented in Figure 8, dem nstrate that baboon serum mitogenic activity is minimally inhibited by MAb 169.31, but inhibited greater than 50% by MAb 163.31. The pool of the two antibodies inhibited greater than 75% of the serum mitogenic activity.

These results demonstrate that the majority of the mitogenic activity in baboon serum towards baboon smooth muscle cells can be inhibited through the use f anti-PDGF receptor monoclonal antibodies. Studies by th inventors have shown that the predominant form of PDGF in baboon platelets is PDGF-BB. Due to the large percentage of PDGF-beta receptors on baboon smooth muscle cells, it is consistent that the anti-PDGF-beta receptor MAb w uld have the largest inhibitory activity towards baboon serum.

Example 10
The Effect of Circulating MAb 169.31 on Baboon Serum Mitogenic Activity

A study was performed to monitor the circulating levels of MAb 169.31 after the administration of a bolus intravenous (i.v.) injection of 25 mg int a baboon. Serum was obtained at various intervals following antibody injection, and the level of circulating antibody was determined by ELISA. Sheep anti-mouse IgG was added to 96-well microtiter dishes in ELISA buffer A at a concentration of 2 μg/ml. The plates were incubated overnight at 4°C, washed with ELISA C buffer, then incubated with ELISA B buffer to block nonspecific binding sites. The plates were washed with ELISA C buffer, then incubated with 100 μl/well of test sample. Baboon plasma or serum containing monoclonal antibody 169.31 was diluted 1:1000 with ELISA B buffer and added to the test wells. Standards, consisting of purified MAb 169.31 spiked int contr 1 baboon plasma or

serum, w re diluted 1:1000, similar t the test plasma/serum sampl s, then added t the test wells. Standards ranged from 100 ng/ml to 1.56 ng/ml final concentration in the test wells. The plasma/serum samples were incubated in the wells for 1-2 hours at 37°C, the wells washed with ELISA C buffer, then goat anti-mouse IgG conjugated with horseradish peroxidase was added. The wells were incubated for 1 hour at 37°C, washed with ELISA C buffer, then incubated with Reaction buffer. The reaction was stopped by the addition of 1 N H_2SO_4, and the plates were read in an ELISA plate reader at 490 nm. ELISA analysis of the baboon serum samples for circulating levels of MAb 169.31 indicated that the *in vivo* half-life of this murine antibody was approximately 15 hours.

In addition, the relative mitogenic potency of the serum samples obtained at 1 hour and 18 hours following the injection was determined. At thess times the circulating levels of antibody in the baboon were determined to be 46 μg/ml and 21 μg/ml, respectively. The 1 hour and 18 hour serum samples were then compared t a control serum sample for relative mitogenic activity. Dilutions of the serum samples were added to baboon vascular smooth muscle cells that had been cultured essentially as descriЬed in the baboon serum study presented above. The final serum concentrations n the smooth muscle cells ranged from 1.25% down to 0.15%. The baboon smooth muscle cells were monitored for the level of [^{3}H] thymidine incorporation, as a means to determine mitogenic activity, essentially as described above.

The results, presented in Figure 9, show that there was a significant decrease in the relative mitogenic potency for both the 1-hour and 18-hour serum samples as compared to the control sample, with the 18-h ur sample being interm diate in mitogenic activity betw en the contr 1 and 1-hour samples. The level of neutralizati n by MAb 169.31 that was present in the 18-

h ur. serum sampl is c nsistent with the level f
neutralizati n obtained when this antibody was added ex
vivo to control baboon serum (Figure 8). Thus, these
results demonstrate that MAb 169.31 circulating f r at
5   least 18 hours in baboon blood retains essentially all of
its biological activity for inhibiting baboon serum
mitogenic activity on baboon smooth muscle cells.

Example 11
10   Inhibition of Cell Outgrowth from Baboon Aortic Explants

Anti-PDGF receptor monoclonal antibodies were
tested for the ability to decrease the rate of smooth
muscle cell outmigration from explants of baboon aortic
15   tissue. The inner media of the thoracic aorta of baboons
was dissected out in DMEM culture media containing 10 mM
Hepes. The aortic tissue was sectioned into 1 mm square
sections, and the explants were placed onto tissue
culture flasks. After a 10 minute incubation to all w
20   time for the explants to adhere to the flasks, culture
media, DMEM plus 6 μg/ml insulin and 5 μg/ml transferin,
was added to the explants, and the samples were incubated
at 37°C with 5% CO_2. A total of 15 explants were set up
in each culture flask. At various times following the
25   establishment of the explants they were examined under a
high power microscope to count the number of explants
that had visible cell outgrowth onto the culture dish.
Explants were counted as positive if at least one cell
migrated from the explant tissue out onto the culture
30   dish surface. Explants were followed for at least seven
days. In experiment #1, the explants were cultured in
the DMEM culture media supplemented with insulin and
transferrin containing the following test samples: 1)
Anti-PDGF alpha receptor MAb (169.31) at 50 μg/ml;   2)
35   Anti-PDGF beta recept r MAb (163.31) at 50 μg/ml; or   3)
DMEM media alone (c ntr 1). In experiment #2 th
explants were cultured in DMEM plus insulin and

transferrin, and eith r 1) a po l of anti-PDGF alpha and
beta rec ptor MAbs (169.31 and 163.31) at 25 µg/ml each;
or 2) DMEM alone (control)

5   The results, presented as the mean percentage
of explants positive for cell outgrowth +/- SEM for each
test condition (Table 5), demonstrate that the anti-PDGF
receptor monoclonal antibodies, individually as well as
in pools, are able to decrease the level of smooth muscle
cell outgrowth from the baboon aortic explants when
10   measured at either four or seven days. These finding
indicate that these antibodies are able to inhibit
processes required for cell migration through a solid
matrix, such as could be required for cells to migrate
through existing vascular tissue toward sites of intimal
15   hyperplasia.

Table 5
Migration of Vascular Smooth Muscle Cells Out of Baboon Aortic Tissue Explants

20

Experiment #1

Sample	(n)	Day 4	Day 7
Control	7	11 +/- 4	61 +/- 4
MAb 169.31	7	6 +/- 2	43 +/- 5
MAb 163.31	7	6 +/- 5	38 +/- 5

Experiment #2

Sample	(n)	Day 4	Day 7
Control	11	9 +/-3	58 +/- 4
Pool	11	2 +/- 1	36 +/- 6

(n): number of times experiment performed.

25

Example 12
Inhibition of PDGF Mitogenic Activity on Human Dermal Fibroblasts by Delayed Addition of Anti-PDGF Receptor Monoclonal Antibodies

Human dermal fibroblasts were plated at approximately 20,000 cells per well in 24-well culture dishes and grown until quiescent in DMEM containing 2% fetal calf serum. The cells were stimulated with either PDGF-AA, AB or BB. Increasing concentrations of each of the PDGF ligands were added to the cells to gen rate standard curves of mitogenic potency for the three PDGF isoforms. The final PDGF concentrations used for the standards were 5, 2.5, 1.25, 0.62, 0.31, 0.15 and 0.0 ng/ml. 50x stock solutions of PDGF were made in 10 mM acetic acid containing 0.25% rabbit serum albumin. 25 μl of each of the stock solutions were added to triplicate test wells. To look for inhibitory activity by MAbs 162.62 and 169.14, wells containing the fibroblasts were incubated with 5 ng/ml of PDGF, final concentration. At various time intervals following the addition of the PDGF samples (1, 2, 4, 6 and 8 hours), a pooled sample of MAb 162.62 and MAb 169.14, 25 μg/ml final concentration f r each MAb, was added to triplicate wells of the cells that had been treated with 5 ng/ml of PDGF. Nine hours after the addition of the PDGF samples, 50 μl of [^{3}H]thymidin , 20 μCi/ml in DMEM containing 1% fetal calf serum, was added to each well. The samples were incubated for an additional 13-15 hours at 37°C. The cells were washed with PBS, then harvested with trypsin and counted in a Betaplate™ liquid scintillation counter (Wallac).

The results, presented in Table 6, are given as mean cpm of [^{3}H]thymidine incorporated +/- standard deviation, f r triplicate determinations. The data are giv n for both the PDGF standard curves, and for the time course f antibody addition. The results demonstrate

that there was a 75% decrease in th mitogenic activity f r PDGF-AA when the anti-PDGF r c ptor antibodies were added to the cells as late as 8 hours following the addition of PDGF ligand. For both PDGF-AB and BB, the addition of the anti-PDGF receptor antibodies 8 hours after the addition of PDGF ligand caused a greater than 90% decrease in PDGF mitogenic activity. These studi s demonstrated that the anti-PDGF receptor monoclonal antibodies can be added to cells at prolonged times after the presence of PDGF ligand and still have potent neutralizing effects against PDGF mitogenic activity.

Table 6

PDGF-AA (ng/ml)			MAbs 162.62/169.14 (PDGF-AA, 5 ng/ml)		
ng/ml	cpm +/-	(st.dev.)	Time in Hrs	cpm +/-	(st.dev.)
5.0	7888	(768)	1	5400	(870)
2.5	7892	(460)	2	4350	(431)
1.25	6044	(1126)	4	5323	(574)
0.62	5569	(315)	6	5300	(768)
0.31	5072	(224)	8	6028	(276)
0.15	4888	(393)			
0.0	4804	(320)			

PDGF-AB (ng/ml)			MAbs 162.62/169.14 (PDGF-AB, 5ng/ml)		
ng/ml	cpm +/-	(st.dev)	Time in Hrs	cpm +/-	(st. dev.)
5.0	16370	(409)	1	4372	(443)
2.5	16621	(878)	2	4783	(401)
1.25	14061	(1066)	4	4363	(427)
0.62	11238	(238)	6	5238	(611)
0.31	9206	(428)	8	5659	(667)
0.15	8061	(1054)			
0.00	5253	(443)			

PDGF-BB (ng/ml)			MAbs 162.62/169.14 (PDGF-BB, 5ng/ml)		
ng/ml	cpm +/-	(st.dev)	Time in Hrs	cpm +/-	(st. dev.)
5.0	12427	(1366)	1	2811	(291)
2.5	15445	(977)	2	3076	(169)
1.25	13712	(976)	4	4298	(574)
0.62	11989	(1248)	6	5089	(420)
0.31	9482	(2089)	8	7335	(502)
0.15	6905	(456)			
0.00	3090	(272)			

Example 13

Displacement of Receptor-bound[125]I-PDGF from Human
Osteosarcoma Cells by Anti-PDGF Receptor MAbs

The four MAbs 162.62, 163.31, 169.14 and 169.31
were analyzed for the ability to displace ^{125}I-PDGF-AA
and ^{125}I-PDGF-BB bound to PDGF receptors on monolayers of
human osteosarcoma cells (ATCC CRL 1427), which express
approximately equal amounts of PDGFr-alpha and PDGFr-
beta. Monolayers of human osteosarcoma cells, grown in
24-well culture plates, were incubated for 1 hour at 4°C
with ^{125}I-PDGF-AA or ^{125}I-PDGF-BB diluted in binding
media. The cells were washed with PBS, then 1 ml of
either binding media alone, MAb 169.14, 169.31, 162.62,
163.31, or a pool of 169.31 and 162.62 was added to each
well. The antibodies were diluted in binding media and
added to the cells at a concentration of 5 µg/ml, 1
ml/well. The cells were washed for 1 hour at 4°C, then
with PBS, incubated with extraction buffer, then
harvested and counted in a gamma counter to monit r the
level of ^{125}I-PDGF binding. 100 ng/ml of PDGF-BB was
added to triplicate wells with ^{125}I-PDGF-AA and [125]I-
PDGF-BB to determine the levels of non-specific binding.
The results, presented in Table 7, are shown as specific
cpm bound (std. dev.) for ^{125}I-PDGF-AA and ^{125}I-PDGF-BB
following the second incubation with the test compounds
listed. The % displacement value was determined by
comparing the cpm bound for the test samples compared to
the cpm bound in the binding medium alone wells. The
results demonstrate that the anti-PDGF alpha receptor
MAbs, 169.14 and 169.31, were able to displace
approximately 63% of the prebound ^{125}I-PDGF-AA. In
contrast, the anti-PDGF beta receptor MAbs, 162.62 and
163.31, had essentially no effect, displacing less than
10% of the c unts. For ^{125}I-PDGF-BB binding, MAbs 169.14
and 169.31 w re able t displace between 22-25% of the

prebound counts while MAb 162.62 was able t displace 34%
of the counts. Th pool of 169.31 and 162.62 displaced
44% of the prebound ^{125}I-PDGF-BB. These results sh w
that, in addition to being able to block PDGF binding,
5 the anti-PDGF receptor MAbs are also able to displace
prebound PDGF-AA and BB from cell-surface receptors.

Table 7

Ability of Anti-PDGF Receptor MAbs to Displace Recptor-
10 bound ^{125}I-PDGF-AA and ^{125}I-PDGF-BB from Human
Osteosarcoma Cells

First Inc.	Second Inc.	CPM Bound	% Displacement
^{125}I-PDGF-AA	Binding Media	458 (46)	0
"	169.14	164 (91)	64
"	169.31	174 (58)	62
"	162.62	415 (18)	9
"	163.31	420 (40)	8
"	169.31/162.62	116 (24)	75
^{125}I-PDGF-BB	Binding Media	528 (41)	0
"	169.14	411 (87)	22
"	169.31	395 (30)	25
"	162.62	349 (48)	34
"	163.31	518 (129)	2
"	169.31/162.62	289 (58)	44

15 ## Example 14

Inhibition of Baboon Serum and PDGF-BB Stimulated Smooth
Muscle Cell Mitogenesis By Anti-PDGFr MAbs Applied
Independently or Coordinately With Heparin

20 Anti-PDGFr-alpha MAb 169.31 and anti-PDGFr-beta
MAb 163.31 w re analyzed independently, and in coordinate

administration assays with heparin, t determine the ability of the antibodies to inhibit PDGF-BB and baboon serum mitogenic activit; on BVSMCs.

To assess the activity of the two antibodies alone, and to assess combinatorial inhibitory activities of the antibodies coordinately administered with heparin, baboon venous smooth muscle cells, at passage 7 foll wing outgrowth, were plated into 24-well tissue culture dishes at 3.0×10^4 cells per well in DMEM (GIBCO BRL) supplemented with 10% fetal bovine serum. The cells were maintained in this medium for three days at 37°C in a 5% CO_2 atmosphere. The medium was then replaced with 1 ml/well of Mito Media, and the cells were cultured for an additional 24 hours.

In a first set of experiments, PDGF-BB was diluted with 10 mM acetic acid containing 0.25% rabbit serum albumin to a concentration of 40 ng/ml. 50 μl of this stock dilution was then added to each well to give a final concentration of 2 ng/ml on the cells. To c rtain of the test wells, unfractionated heparin (UH) (Sigma Chemical Co., St. Louis, MO) was added alone or coordinately administered with one or more of the anti-PDGFr antibodies. The UH employed for these studi s was a mixture of heparin species of multiple sizes, with a specific activity of appproximately 150 Units/mg in a standard Activated Partial Thromboplastin Time (APTT) assay. The addition of heparin to the cells was d ne by diluting the heparin to a stock concentration of 400 μg/ml in PBS, and adding 25 μl of the heparin solution t the appropriate wells to give a final h parin concentration of 10 μg/ml on the cells. The anti-PDGFr antibodies were diluted with PBS to give 40x stock concentrates, then 25 μl of the antibody dilution was added to appropriate test wells. Those wells receiving only antibody r h parin independently receiv d 25 μl of PBS as a buffer control.

A d se-response profile f r PDGF-BB stimulation was generated by making 2-fold dilutions of the PDGF with 10 mM acetic acid containing rabbit serum albumin and adding 50 μl of the stock solutions to appropriate w lls

5   to give final PDGF-BB concentrations on the cells of 2, 1 and 0.5 ng/ml.

After addition of the treatments, the cells were incubated for 20 hours at 37°C. Mitogenic stimulation of BVSMCs was assessed by measuring the

10 uptake of [^{3}H]thymidine. 50 μl of a 20 μCi/ml [^{3}H]thymidine stock solution, made up in DMEM, was added directly to the cells for a final concentration f 1 μCi/well. The cells were incubated for 4 hours at 37°C, washed once with PBS, treated with 0.25 ml of trypsin

15 until cells detached, and harvested onto a filter using a cell harvester (LKB Wallac). The filters were c unted using a Betaplate™ liquid scintillation counter (Wallac).

The data for antibody and antibody/heparin

20 inhibition of PDGF-BB stimulation are shown in Table 8. Data in Table 8 are presented as mean counts per minute (cpm) of [^{3}H]thymidine incorporated by baboon sm th muscle cells stimulated with PDGF-BB. Values of percent inhibition were determined directly from the measured

25 decrease in incorporation of [^{3}H]thymidine. A dose-response table of PDGF-BB mitogenic activity is included in Table 8. In Experiment #1, the addition of antibody 169.31 to cells stimulated with PDGF-BB caused a marked inhibition in [^{3}H]thymidine incorporation at antibody

30 doses of 1 and 0.1 μg/ml. The administration of heparin to the cells coordinately with antibody 169.31 resulted in a combinatorially effective antimitogenic result, i.e. [^{3}H]thymidine incorporation was inhibited to a greater extent than was measured for either the antibody or

35 heparin administered alone. Analysis of antibody 163.31, Experiment #1, show d that a dose of 25 μg/ml was also able to inhibit [^{3}H]thymidine inc rporation, but at a

much lower level than observed f r antibody 169.31. When h parin was added along with antibody 163.31, a combinatorially effective inhibition was also observed, but this effect was only seen at higher antibody concentrations. The coordinate application of antibodies 169.31 (1 μg/ml) and 163.31 (25 μg/ml) also resulted in a combinatorially antimitotic result, wherein inhibiti n of PDGF-BB-stimulated [^{3}H]thymidine incorporation by the BVSMCs was greater than that observed following administration of either antibody alone.

In Experiment #2, antibodies 169.31 and 163.31 were further analyzed, both independently and in coordinate administration assays with one another or with heparin (UH, 10 μg/ml), to determine the anti-mitotic activities of these various treatments on PDGF-BB-stimulated [^{3}H]thymidine incorporation by BVSMCs. Similar to the results of Experiment #1, the coordinate administration of the two antibodies provided mor effective inhibition of mitogenic activity on BVSMCs than administration of either antibody alone. In additi n, coordinate administration of heparin with a pool of the two antibodies led to combinatorially effective inhibition above inhibitory activity provided by the heparin or antibody pool alone.

In sum, these data demonstrate that the coordinate administration of heparin with either of the anti-PDGFr-alpha or anti-PDGFr-beta antibodies or with a pool of the two antibodies, as well as the coordinate administration of the antibodies with one another, can provide a combinatorially effective treatment against PDGF-BB mitogenic activity on BVSMCs.

Table 8

Inhibition of PDGF-BB Mitogenic Activity By Anti-PDGFr MAbs Administered Independently or Coordinately With Heparin

EXPERIMENT 1

MAb (µg/ml)		[³H]Thymidine Incorporation			
163.31	169.31	(-) Heparin	% Inhib.	(+) Heparin	% Inhib.
0	0	4,714	0%	2,543	46%
0	1	2,271	52%		
0	0.1	2,637	44%	1,582	66%
0	0.01	4,078	13%	1,983	58%
0	0.001	4,486	05%	2,311	51%
25	0	3,969	16%	1,844	61%
5	0	4,460	05%	2,484	47%
1	0	4,268	09%	2,559	46%
0.2	0	4,504	04%	2,671	43%
25	1	1,015	78%		
5	1	1,859	61%		
1	1	2,007	57%		
1	0.2	1,979	58%		
1	0.04	2,090	56%		
0	0	4,414	06%		

PDGF-BB Dose-Response

PDGF-BB (ng/ml)	CPM
2.0	4,714
1.0	2,313
0.5	1,588
0.0	161

Table 8. continued

EXPERIMENT 2

MAb (μg/ml)		[³H]Thymidine Incorporation			
163.31	169.31	(-) Heparin	% Inhib.	(+) Heparin	% Inhib.
0	0	3,469	0%	2,116	39%
0	1	2,283	34%		
0	0.1	2,183	37%		
0	0.01	2,778	20%		
25	0	2,139	38%		
5	0	3,425	01%		
1	0	3,958	0%		
25	1	797	77%	540	85%
5	1	1,595	54%	999	71%
1	1	1,976	43%	1,201	65%
0	1	2,283	34%	1,551	55%

5   Inhibition of the mitogenic activity of babo n serum was tested in a parallel set of experiments. The experiments were performed essentially as described above, with baboon serum added to the test wells at a final concentration of 1.25% by diluting the serum 1:4

10   with PBS and adding 50 μl of the diluted serum to each well. A dose-response profile for baboon serum stimulation was generated by making 2-fold diluti ns f the baboon serum in PBS and adding 50 μl of the appropriate dilutions to give final serum concentrations

15   on the cells of 1.25, 0.62, 0.31 and 0.15%.

      The data for antibody and antibody/heparin inhibition of serum mitogenic activity on BVSMCs are presented in Table 9. These data show that monoclonal antibody 169.31 had minimal inhibitory effect on

20   [³H]thymidine incorporation stimulated by the addition of 1.25% baboon serum, at d ses of antibody up t 1 μg/ml. When heparin was coordinately administered t the cells

al ng with antibody 169.31, the level f inhibition
bserved was n gr ater than the inhibit ry activity
exhibited by heparin alone. In contrast, the assays
involving monoclonal antibody 163.31 showed significant
5   inhibition of [^{3}H]thymidine incorporation at an antibody
concentration of 25 μg/ml. Moreover, when heparin was
administered coordinately with antibody 163.31, there was
a marked combinatorially effective inhibition of
mitogenic activity as measured by [^{3}H]thymidine
10   incorporation, i.e. well above the antimitogenic effects
observed for either the antibody or heparin alone. This
combinatorially effective inhibition was particularly
pronounced at MAb 163.31 concentrations between 0.2 μg/ml
and 5 μg/ml.

15   Coordinate administration of 1 μg/ml of
antibody 169.31 with increasing doses of antibody 163.31
showed a dose-dependent inhibition of [^{3}H]thymidine
incorporation. The coordinate administration of antibody
169.31 with antibody 163.31 also resulted in a pronounced
20   combinatorially effective inhibition of serum mitogenic
activity as shown in Table 9.

          Data in Table 9 are presented as mean counts
per minute (cpm) of [^{3}H]thymidine incorporated by baboon
smooth muscle cells stimulated with 1.25 % baboon serum.
25   Values of percent inhibition were calculated from a
standard curve generated using the serum dose-response
data presented.

Table 9

Inhibition of Baboon Serum Stimulated Smooth Muscle Cell Mitogenesis By Anti-PDGFR MAbs Administered Independently or Coordinately With Heparin

MAb (ug/ml)		[3H]Thymidine Incorporation			
163.31	169.31	(-) Heparin	% Inhib.	(+) Heparin	% Inhib.
0	0	1,297	0%	957	31%
0	1	1,028	24%		
0	0.1	1,242	4%	924	33%
0	0.01	1,114	16%	855	38%
0	0.001	1,482	0%	922	33%
25	0	591	57%	451	73%
5	0	924	33%	474	70%
1	0	895	35%	587	59%
0.2	0	980	28%	651	50%
25	1	362	82%		
5	1	481	70%		
1	1	598	56%		
1	0.2	761	44%		
1	0.04	893	35%		

Serum Dose-Response

(%NBS)	(CPM)
1.25	1,297
0.62	639
0.31	410
0.15	317
0	161

Example 15
Inhibition of Serum Mitogenic Activity on Baboon Vascular Smooth Muscle Cells By Heparin Administered Alone, or Coordinately Administered With Anti-PDGFr MAbs

Both unfractionated heparin (UH) (Sigma, St. Louis, MO) and low molecular weight heparin (LMWH) (Logiparin®, Novo Nordisk, Bagsvaerd, Denmark) were evaluated for their ability to inhibit the mitogenic activity of baoon serum on baboon vascular smooth muscle cells. Each form of heparin was administered to the cells independently or coordinately with anti-PDGFr MAbs. The LMWH used for these studies was generated by heparinase treatment of an unfractionated heparin, and is composed of heparin species with an average molecular weight of 5,500 daltons. The LMWH has a decreased antithrombotic activity compared to unfractionated heparin in an APTT assay, estimated at about 50 Units/mg.

To assess the anti-mitogenic activity of the two heparin preparations, with and without coordinately administered anti-PDGFr antibodies, BVSMCs from a rtic explants (designated BO54 cells) were plated into 24-well tissue culture dishes at 2.5×10^4 cells per well in DMEM (GIBCO BRL) supplemented with 10% fetal bovine serum. The cells were maintained in this media for three days at 37°C in a 5% CO_2 atmosphere. The media was then replaced with 1 ml/well of Mito Media, and the cells were cultur d in this media for an additional 24 hours.

Baboon serum was added to the test wells at a final concentration of 2.5%. This was done by diluting the serum 1:1 with PBS and adding 50 μl of the diluted serum to each well. To analyze heparin preparations for their inhibition of baboon serum-stimulated DNA synthesis in baboon smooth muscle cells, the heparin was add d to the cells eith r independantly or coordinately with the two anti-PDGFr mon clonal antibodies. The heparin samples were diluted with PBS to give a 400 μg/ml

c ncentrat , th n 25 μl f the c ncentrate was added t ach test well to give a final heparin concentration of 10 μg/ml. Control wells received phosphate buffered saline only. Anti-PDGFr-alpha MAb 169.31 and anti-PDGFr-

5 beta MAb 163.31 were diluted with PBS to 40 μg/ml and 1 mg/ml, respectively, and 25 μl of the diluted antibodies was added to appropriate test wells to give final antibody concentrations of 1 μg/ml for MAb 169.31 and 25 μg/ml for MAb 163.31.

10   After the addition of the treatments, the cells were incubated for ᴢ0 hours at 37°C. Mitogenic stimulation of the smooth muscles cells was assessed by measuring cellular incorporation of $[^3H]$thymidine as disclosed in Example 14.

15   The results of this study are presented in Table 10. Those cells treated with serum only in the absence of either heparin or anti-PDGFr MAbs had a control value for $[^3H]$thymidine incorporation of 36,032 cpm/well. Treatment of the cells with coordinately

20 administered anti-PDGFr-alpha and anti-PDGFr-beta monoclonal antibodies caused a decrease in $[^3H]$thymidine incorporation to 27,000 cpm (i.e. a 25 % inhibition of serum-stimulated DNA synthesis). There was no significant reduction in $[^3H]$thymidine incorporation when

25 the cells were treated with either UH or LMWH alone. However, a significant decrease in $[^3H]$thymidine incorporation was observed when either the UH or the LMWH was added to the cells coordinately with the pool of anti-PDGFr antibodies. The data demonstrate that both UH

30 and LMWH, when coordinately administered with a po l of anti-PDGFr-alpha and anti-PDGFr-beta antibodies, inhibit the mitogenic activity of autologous serum on BVSMCs in a combinatorially effective manner. Here again, the degree of combinatorially effective inhibition achi v d

35 demonstrates a synergistic or p tentiating relationship between the antibody and heparin in the particular coordinate administrati n regime tested.

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Antibody Treatment	(-) Heparin	(+)UH	(+)LMWH
Control	36,032 +/- 4,512	34,912 +/- 5,617	34,140 +/- 2,667
(% Inhibition)		(3%)	(5%)
Anti-PDGFR	27,005 +/- 2,227	12,377 +/- 3,785	19,967 +/- 974
(% Inhibition)	(25%)	(66%)	(45%)

Table 10

Data are presented as mean +/- standard deviation for
counts per minute (CPM) of [^{3}H]thymidine incorporated by
baboon smooth muscle cells stimulated with 2.5% baboon
serum. Percent inhibition of cpm incorporated from
control value, in the absence of both added heparin and
anti-PDGFR antibody, is presented in parentheses. Values
of percent inhibition were determined directly fr m the
measured decrease in incorporation of [^{3}H]thymidine,
rather than by comparison to a dose-response curve for
serum stimulation.

Example 16
Dose-Response of Inhibition of Serum Mitogenic Activity
On Baboon Vascular Smooth Muscle Cells By Anti-PDGFr MAbs
Coordinately Applied With Unfractionated Heparin or Low
Molecular Weight Heparin

A dose-response assay of anti-PDGFr beta
monoclonal antibody 163.31, in the presence of a constant
amount of anti-PDGFr alpha antibody 169.31 (1 μg/ml), was
performed to evaluate concentration dependence of anti-
PDGFr MAb inhibition of DNA synthesis in BVSMCs
stimulated by baboon serum. This dose-response was

evaluated in the absence of any added heparin, as well as in the presence of 10 μg/ml f either an unfracti nated heparin (UH) with an antithrombtic activity of about 150 Units/mg by APPT assay (Sigma Chemical Co., St. Louis, MO) or a low molecular weight heparin (LMWH) (Logiparin*, approximately 50 Units/mg by APPT assay) (Novo Nordisk, Bagsvaerd, Denmark).

For these dose-response studies, BVSMCs fr m aortic explants (BO54 cells) were plated into 24-well tissue culture dishes at 2.5 x 10^4 cells per well in DMEM supplemented with 10% fetal bovine serum. After 3 days the media was changed to Mito Media, and the cells were cultured for an additional 24 hours. The cells were stimulated to undergo mitosis by the addition of 50 μl/well of baboon serum that had been diluted 1:1 with PBS, giving a final serum concentration of 2.5% n the cells. MAb 163.31 was diluted with PBS to make 40x concentrates, and 25 μl of the diluted antibody was added to the test wells to give final antibody concentrations on the cells ranging from 25 μg/ml to 1.25 μg/ml. 25 μl of a 40 μg/ml solution of MAb 169.31 was simultane usly added to the wells to give a final antibody concentrati n of 1 μg/ml in all of the 163.31 treated wells. The combination of monoclonal antibodies 163.31 and 169.31 was tested in the absence of any added heparin and, alternatively, in a coordinate administration assay with either UH or LMWH. The two heparin preparations w re diluted with PBS to a final concentration of 400 μg/ml, and 25 μl of concentrate was added to appropriate wells to give a final heparin concentration on the cells of 10 μg/ml. PBS only was added to those test wells not receiving heparin.

After the addition of the treatments, the cells were incubated for 20 hours at 37°C. Mitogenic activity was assessed by measuring the uptake of [^{3}H]thymidine as discl sed in Example 14.

The results f this study are presented in Table 11. In the absence f heparin, a significant decrease in mitotic activity of the BVSMCs was observed only in those wells receiving the 25 and 10 μg/ml doses

5 of monoclonal antibody 163.31. However, in the presence of either UH or LMWH, all concentrations of monoclonal antibody 163.31 tested (in the presence of a constant amount of MAb 169.31) provided significant combinatorially effective inhibition, with essentially

10 identical results obtained for the two heparin preparations. In the presence of heparin, the 1.25 μg/ml dose of 163.31 was more effective at inhibiting DNA synthesis than the 25 μg/ml dose of 163.31 in the absence of heparin. The presence of either UH or LMWH

15 administered independent of any antibody had only a minimal effect on the mitogenic activity of serum.

Table 11

MAb (μg/ml)		CPM ± Std.Dev. (% Inhibition)		
163.31	169.31	(-) Heparin	(+) UH	(+) LMWH
0	0	31,002 ± 2,655	27,175 ± 1,518 (12%)	27,573 ± 947 (11%)
25	1	23,424 ± 371 (24%)	11,811 ± 365 (62%)	13,095 ± 409 (58%)
10	1	23,901 ±4,138 (23%)	12,317 ± 2,034 (60%)	11,829 ± 1,451 (62%)
5	1	29,366 ±1,652 (5%)	18,449 ± 1,802 (40%)	18,832 ± 1,530 (39%)
2.5	1	27,192 ± 2,351 (12%)	16,249 ± 4,075 (48%)	16,720 ± 2,674 (46%)
1.25	1	29,032 ± 1,012 (6%)	19,663 ± 1,455 (37%)	21,664 ± 1,485 (31%)

Table 11, continued

> Data are present d as mean ± standard deviation
> for counts per minute (CPM) of [^{3}H]thymidine
> incorporated by baboon smooth muscle cells
> stimulated with 2.5% baboon serum. UH =
> Unfractionated heparin, LMWH = Low molecular
> weight heparin. Values of percent inhibition
> were determined directly from the measured
> decrease in incorporation of [^{3}H]thymidine.

Example 17

Comparison of Antimitogenic Activities of Murine Anti-PDGFr-Alpha MAb 169.31 With Mouse/Human Chimeric Anti-PDGFr-Alpha Antibody

A mouse/human chimeric antibody was generated
using the anti-PDGFr-alpha monoclonal antibody 169.31 as
the parent antibody for cloning out the light and heavy-
chain variable domains.   This mouse/human chimeric
antibody comprises the variable domains of the parent
murine monoclonal antibody and the constant domains for
human IgG4 heavy-chain and human kappa light-chain.
Construction of this antibody used standard techniques as
described in Mountain and Adair, Biotech. and Genet. Eng.
Rev. 10: 1-142, 1992; and Adair et al, Immunol. Rev. 130:
5-40, 1992.   The parent murine antibody and the
mouse/human chimeric antibody were directly compar d f r
their ability to inhibit DNA synthesis of BVSMCs (BO54)
stimulated with 2% baboon serum.

The parent murine and the chimeric anti-PDGFr-
alpha antibodies were analyzed at both 1.0 and 0.1 µg/ml
in the presence of 10 µg/ml of murine anti-PDGFr-beta
antibody 163.31 and 10 µg/ml of an unfractionated heparin
(Elkins-Sinn, Inc., Cherry Hill, N.J.; specific activity
≅150 units/mg).   In addition, b th anti-PDGFr-alpha
antibodies (1 µg/ml) wer added t the cells in the

presence of 10 μg/ml of chimeric anti-PDGFr-beta antibody
(see Example 18) and 10 μg/ml f heparin. The r sults,
presented in Table 12, demonstrate that both the parent
murine MAb 169.31 and the mouse/human chimeric anti-
5 PDGFr-alpha antibody have similar inhibitory potency in
the presence of either the parent murine anti-PDGFr-beta
MAb 163.31 or the mouse/human chimeric anti-PDGFr-beta
antibody.

10

TABLE 12

Antimitogenic Activities of Parent Murine and Mouse/Human Chimeric Anti-PDGFr-Alpha Antibodies Coordinately Administered With Heparin

Serum (2%)	Anti-PDGFr alpha (μg/ml)	Anti-PDGFr beta (10 μg/ml)	Heparin	CPM ± S.D.
+	Buffer	Buffer	No	7,242 ± 329
-	Buffer	Buffer	No	71 ± 6
+	169.31 (1.0)	163.31	Yes	2,278 ± 321
+	169.31 (0.1)	163.31	Yes	3,225 ± 366
+	Ch 169 (1.0)	163.31	Yes	2,879 ± 620
+	Ch 169 (0.1)	163.31	Yes	3,279 ± 985
+	169.31 (1.0)	Ch 163	Yes	2,715 ± 170
+	Ch 169 (1.0)	Ch 163	Yes	2,600 ± 575

15

Data are presented as counts per minute (cpm) ± standard
deviation of [^{3}H]thymidine incorporated by baboon SMCs
following stimulation with 2% baboon serum. Ch 169 =
Mouse/human chimeric anti-PDGFr-alpha antibody, Ch 163 =
20 Mouse/human chimeric anti-PDGFr-beta antibody.

Example 18
Comparison of Antimitogenic Activities of Murine Anti-
PDGFr-Beta MAb 163.31 With Mouse/Human Chimeric Anti-
PDGFr-Beta Antibody

A mouse/human chimeric anti-PDGFr-beta antibody was generated using the murine anti-PDGFr-beta monocl nal antibody 163.31 as the parent antibody for cloning ut the light and heavy-chain variable domains. This mouse/human chimeric antibody comprises the variable domains of the parent murine monoclonal antibody and the constant domains for human IgG4 heavy-chain and human kappa light-chain. Construction of this antibody used the same standard techniques for chimeric antibody construction as described above for the chimeric anti-PDGFr-alpha antibody (Example 17). The parent murine antibody and the mouse/human chimeric antibody were directly compared for their ability to inhibit DNA synthesis in baboon smooth muscle cells stimulated with 2.0% baboon serum.

BVSMCs (BO54) were plated at a density of 2 x 10^4 cells/ well in 24-well culture plates and grown for approximately 48 hours in DMEM containing 10% fetal calf serum at 37°C. The rells were then incubated for 24 hours in Mito Media to allow them to become quiescent.

A control plate of cells was stimulated with a two-fold dilution series of normal baboon serum (2.0% to 0.125%) to produce a standard curve. The serum was diluted in PBS, and 50 μl of a 20X stock was added directly to the wells in triplicate.

A dilution series of the anti-PDGFr-beta MAb 163.31, or the chimeric anti-PDGFr-beta antibody, was administered coordinately in appropriate test wells with the anti-PDGFr-alpha MAb 169.31 (1 μg/ml) and unfracti nated heparin (2 Units/ml) (Elkins-Sinn, Inc., Cherry Hill, N.J.) t assess inhibitory effects of these treatments on BVSMCs stimulated by 2.0% baboon serum. In

addition, the anti-PDGFr-beta parent and chimeric antibodies were analyzed in independent administrati n assays and in coordinate administration assays using the antibody and heparin. The antibodies and heparin were
5   added to appropriate test wells at 25 μl per well of a 40 X stock diluted in PBS. The antibody concentrations used are indicated in Table 13.

After administration of the test samples, the cells were incubated for 18 hours at 37°C. Mitogenic
10   activity was assessed by uptake of [^{3}H] thymidine.

The results of the anti-PDGFr-beta MAb dose-response experiment, presented in Table 13, demonstrate that baboon serum-induced mitogenic activity was inhibited approximately 80% by a combination of 25 μg/ml
15   163.31, 1 μg/ml 169.31 and 2 Units/ml heparin. Similar results were obtained using the chimeric anti-PDGFr-beta MAb. Coordinate administration of each of the murine and chimeric anti-PDGFr-beta MAb, at 25 μg/ml, with the anti-PDGFr-alpha MAb 169.31, resulted in approximately 30%
20   inhibition of baboon serum mitogenic activity. The anti-PDGFr-beta MAbs coordinately administered with heparin produced roughly 40% inhibition. Each of the antimitogenic agents, when administered independ ntly, resulted in less than 30% inhibition of baboon serum
25   mitogenic activity.

These results demonstrate that the mouse/human chimeric anti-PDGFr-beta antibody has similar inhibitory activity as that of the parent murine anti-PDGFr-beta antibody MAb 163.31. This activity provid s a
30   combinatorially effective inhibition of serum mitogenic activity on BVSMCs when the chimeric anti-PDGFr-beta antibody is administered coordinately with either the anti-PDGFr-alpha MAb 169.31, heparin, or a combination of anti-PDGFr-alpha MAb and heparin.

Table 13

Comparison of Antimitogenic Activities of Murine Anti-PDGFr-Beta MAb 163.31 and Mouse/Human Chimeric Anti-PDGFr-Beta in Independent and Coordinate Administration Assays With Anti-PDGFr-Alpha MAb and Heparin

MAb (µg/ml)		CPM ± S.D. (Percent Inhibition)	
163.31	169.31	(-) Heparin	(+) UH
0	0	7539 ± 554 (0%)	5,975 ± 383 (27%)
25	0	6,580 ± 449 (18%)	4,786 ± 423 (41%)
0	1	8,802 ± 641 (0%)	6,479 ± 1,120 (20%)
1.56	1	_____	3,973 ± 588 (52%)
6.25	1	_____	2,861 ± 177 (66%)
25	1	5,791 ± 957 (29%)	1,495 ± 16 (83%)

Antibody (µg/ml)		CPM ± S.D. (Percent Inhibition)	
Chimeric 163	Mab 169.31	(-) Heparin	(+) UH
0	0	7539 ± 554 (0%)	5,975 ± 383 (27%)
25	0	6,721 ± 0 (16%)	4,878 ± 427 (40%)
0	1	8,802 ± 641 (0%)	6,479 ± 1,120 (20%)
1.56	1	_____	4,528 ± 899 (45%)

Table 13, continued

6.25	1	_____	2,892 ± 382
			(65%)
25	1	5,164 ± 519	1,973 ± 374
		(37%)	(77%)

Data are presented as the counts per minute (cpm) ±
standard deviation of $[^3H]$thymidine incorporated by
5   baboon SMCs following stimulation with 2% baboon serum.
Values of percent inhibition for both the parent and
chimeric antibody studies were determined by comparing
the cpm incorporated of $[^3H]$thymidine to a standard curve
generated from the serum dose-response data presented
10 below.

Serum Dose-Response

(%NBS)	(CPM+/-S.D.)
0	89 ± 0
0.125	444 ± 89
0.25	978 ± 62
0.5	2,190 ± 46
1	4,655 ± 248
2	7,768 ± 585

Example 19

15 **Dose-Responsive Inhibition of Serum Mitogenic Activity on
Baboon Vascular Smooth Muscle Cells by Heparin
Administered Independently or Coordinately With Anti-
PDGFr Antibodies**

20   Baboon smooth muscle cells (BO54) were plated
at a density of 2×10^4 cells/well in 24-well culture
plates and grown for approximately 72 hours in DMEM
containing 10% fetal calf serum at 37°C. The cells were
then made quiescent by incubating them for 24 hours in
25 Mito Media. The ability of heparin alone t inhibit
mitogenic stimulation by 2% baboon serum was test d by

adding a dilution seri s of either an unfractionated heparin (UH; approximately 150 Units/mg) (Elkins-Sinn, Inc.) or low molecular weight heparin (LMWH; Logiparin , approximately 50 Units/mg) (Novo Nordisk, Bagsvaerd,
5 Denmark) to the cells at doses ranging from 15 U/ml t 0.06 U/ml (final concentation on the cells). The same dilution series for both types of heparin was also tested in the presence of anti-PDGFr-beta MAb 163.31 (10 μg/ml) and anti-PDGFr-alpha MAb 169.31 (1 μg/ml).

10 After the addition of the treatments the cells were incubated for 18 hours at 37°C. Serum mitogenic activity was assessed by measuring uptake f $[^3H]$thymidine. The results of the heparin d se-response experiments are presented in Table 14. Heparin
15 alone at the highest doses tested had only a modest inhibitory effect on $[^3H]$thymidine incorporation by BVSMCs, while the same doses coordinately administered with the anti-PDGF receptor antibodies combinatorially inhibited up to 90% of the serum mitogenic activity.
20 Assuming a specific activity of 150 U/mg for the Elkins-Sinn unfractionated heparin, and 50 U/mg for the low molecular weight heparin, the highest doses used f r the two heparin preparations were about 100 and 300 μg/ml, respectively. At these concentrations in the independent
25 administration assays there was only minimal inhibiti n of mitogenic activity. In contrast, at heparin d ses 100-fold lower there was still a significant combinatorially effecti:e increase in inhibitory activity when the heparin was coordinately administered with the
30 anti-PDGFr antibodies. These results demonstrate that significantly lower doses of heparin can be used t act in a combinatorially effective fashion with the anti-PDGFr antibodies to inhibit $[^3H]$thymidine incorporation, well above levels of inhibition achieved with the
35 antibodies or heparin al ne.

Table 14

Dose-Responsive Inhibition of Serum Mitogenic Activity by Heparin Administered Independently or Coordinately With Anti-PDGFr Antibodies

| | | | (+) MAb 163.31 (10 µg/ml) | |
| | | | (+) MAb 169.31 (1 µg/ml) | |
UH (U/ml)	cpm	Std.Dev.	cpm	Std.Dev.
0	4,694	586	3,532	276
0.06	5,816	495	2,662	377
0.18	4,653	368	2,030	246
0.55	4,900	527	1,133	157
1.67	4,473	405	714	56
5	4,451	357	526	43
15	3,628	255	369	16

LMWH (U/ml)	cpm	Std.Dev.	cpm	Std.Dev.
0	5,715	665	2,904	28
0.06	5,169	438	2,355	301
0.18	4,535	728	1,038	124
0.55	4,210	392	1,151	60
1.67	4,207	2422	641	9
5	3,642	734	678	52
15	3,457	369	380	35

Example 20

Inhibition of Smooth Muscle Cell Outmigration from Baboon Aortic Explants by Coordinate Administration of Anti-PDGFr MAbs and Heparin

Anti-PDGFr monoclonal antibodies were further tested in the presence or absence of heparin for their ability to decrease rates of baboon vascular smooth muscle cell outmigration from explants of baboon aortic tissue. Baboon aortic explants were set up essentially as described in Example 11. The explants were cultured in DMEM supplemented with insulin and transferin and containing the following test samples: 1) Anti-PDGFr-alpha MAb (169.31) and anti-PDGFr-beta MAb (163.31), each antibody at 25 µg/ml, 2) Unfractionated heparin (Sigma

Chemical C .) (100 μg/ml), 3) Anti-PDGF alpha receptor MAb (169.31) and anti-PDGF beta receptor MAb (163.31) (25 μg/ml each) and unfractionated heparin (100 μg/ml), and 4) DMEM (control).

5   The results, presented in Table 15, demonstrate that, when measured at 7 days following establishment of the explants, heparin alone decreased the level of smooth muscle cell outmigration to 82% of control, while the anti-PDGF receptor antibody combination decreased th outgrowth to 64% of control. Coordinate administrati n of both the anti-PDGF receptor antibodies and heparin further decreased the level of outmigration to 42% of control. Thus, heparin and the anti-PDGF receptor antibodies combinatorially inhibited smooth muscle cell outmigration in the coordinate administration regimes tested.

TABLE 15
Inhibition of Smooth Muscle Cell Outgrowth from Baboon Aortic Tissue Explants by Combination of Anti-PDGFr MAbs and Heparin

Condition	n	Mean	St.Dev.	% Control
Control	7	65.6	16.8	___
Anti-PDGFr	7	41.9	22.1	63.8
Heparin	7	53.7	17.2	81.9
Heparin + Anti-PDGFr	7	27.7	13.5	42.3

Example 21
Saturation Binding Analysis of MAb 163.31 on B054 Cells in the Presence and Absence of Heparin

The binding ability of anti-PDGFr-beta MAb 163.31 on babo n sm oth muscl cells (B054) was tested in

the pr sence and absenc of heparin. Baboon smooth muscle cells (BO54) were plated at a density of 20,000 cells/well in 24-well culture plates and grown for approximately 48 hours at 37°C in DMEM containing 10% fetal calf serum. The cells were incubated for 24 h urs in Mito Media to become quiescent, then washed once with 4°C binding media (DMEM/Hams F-12, 25mM Hepes, 0.1%BSA). The binding activity of MAb 163.31 on PDGF-beta receptors was analyzed in the presence of heparin by adding four-fold dilutions of ^{125}I-labeled MAb 163.31 (^{125}I-163.31) in 4°C binding media (ranging from 2.1 x 10^6 to 1 x 10^3 cpm/ml) and 10 μg/ml heparin (Elkins-Sinn, Inc.) to the appropriate wells in triplicate in 1 ml aliquots. On a separate plate, the same dilution series of ^{125}I-163.31 was added without heparin. To determine the level of nonspecific binding by ^{125}I-163.31, a set of triplicate wells was. set up on each plate containing 5.5 x 10^5 cpm/well of the ^{125}I-163.31 plus 25 μg/ml unlabeled 163.31. The plates were kept on ice while samples were being added, then incubated for 1.5 hour at 4°C on a rotary shaker. After washing 3x with PBS, the cells were incubated with an extraction buffer (PBS, 1% NP-40), and the extracts were harvested to 12 x 75 mm tubes and counted in a gamma counter. The results of the binding studies, presented in Table 16, demonstrate that the coordinate addition of heparin and antibody had no significant effect on antibody binding. Thus, the combinatorial effectiveness of coordinately administering heparin with the anti-PDGFr-beta antibodies, shown in the above examples, does not appear to be attributable to any stimulation by heparin of binding of the antibody to cell-surface PDGF-beta receptors.

Table 16
Saturation Binding Analysis of MAb 163.31 on Baboon
Vascular Smooth Muscle Cells in the Presence and Absence
of Heparin

Mean Specific cpm +/- Std.Dev.

^{125}I-163.31

Applied cpm	(+) Heparin	(-) Heparin
2,100,000	8,956.0 +/- 678.1	9,235.0 +/- 632.3
555,000	2,036.0 +/- 62.8	2,301.0 +/- 329.0
148,000	604.0 +/- 56.5	685.0 +/- 8.5
46,000	165.2 +/- 12.3	212.0 +/- 16.6
11,000	84.7 +/- 9.0	81.0 +/- 3.8

Example 22
Saturation Binding Analysis of PDGF-BB on Baboon Vascular
Smooth Muscle Cells in the Presence and Absence of
Heparin

The ability of PDGF-BB to bind its receptor on baboon vascular smooth muscle cells was tested in the presence and absence of heparin. Baboon smooth muscle cells (BO54) were plated at a density of 20,000 cells/well in 24-well culture plates and grown for approximately 48 hours at 37°C in DMEM containing 10% fetal calf serum. The cells were then made quiescent by incubating them for 24 hours in Mito Media, then washed once with 4°C binding media. The binding activity of the PDGF-BB to its receptors was analyzed in the presence of heparin by adding two-fold dilutions of ^{125}I-PDGF-BB in 4°C binding media (ranging from 2 x 10^5 to 2.5 x 10^4 cpm/ml) containing 10 µg/ml heparin (Elkins-Sinn, Inc.) t the appr priate wells in triplicate in 1 ml aliquots.

On a separat plate, th sam dilution series of ^{125}I-PDGF-BB was added without heparin. A set of triplicate wells on each plate also contained 2.0 x 10^5 cpm/well of ^{125}I-PDGF-BB in addition to 1 $\mu g/ml$ unlabeled PDGF-BB to

5   determine the level of nonspecific binding by ^{125}I-PDGF-BB. The plates were kept on ice while samples were being added, and then incubatzd for 1.5 hour at 4°C on a r tary shaker. After washing 3x with PBS, the cells were incubated with an extraction buffer, and the extracts

10   were harvested to 12 x 75 mm tubes and counted in a gamma counter.

       The results, presented in Table 17, demonstrate that the presence of heparin had no significant effect n ^{125}I-PDGF-BB binding. Thus, the combinatorial

15   effectiveness of coordinately administering heparin with the anti-PDGFr antibodies, shown in the above examples, does not appear to be attributable to any inhibition by heparin of the binding of PDGF-BB to cell-surface PDGF receptors.

20

Table 17

Saturation Binding Analysis of PDGF-BB on Baboon Vascular Smooth Muscle Cells in the Presence and Absence of Heparin

25

^{125}I PDGF-BB (applied cpm)	Mean Specific cpm +/- Std.Dev.	
	(+) Heparin	(-) Heparin
200,000	1,674.0 +/- 15.7	1,800.0 +/- 112.0
100,000	1,428.5 +/- 61.0	1,572.0 +/- 80.6
50,000	1,327.0 +/- 91.3	1,438.5 +/- 41.2
25,000	1,005.4 +/- 63.7	1,107.8 +/- 58.2

Example 23
Inhibition of PDGF-BB Binding to Smooth Muscle Cells By MAb 163.31 in the Presence and Absence of Heparin

5   An experiment was carried out to determine th ability of MAb 163.31 to inhibit ^{125}I-PDGF-BB binding t the PDGF beta-receptor on baboon vascular smooth muscle cells in the presence and absence of heparin.

          Baboon smooth muscle cells (BO54) were plated
10   at a density of 20,000 cells/well in 24-well culture plates and grown for approximately 48 hours at 37°C in DMEM containing 10% fetal bovine serum. The cells were incubated for 24 hours in Mito Media to become quiescent, then washed once with 4°C binding media. MAb 163.31 was
15   diluted in binding media to the concentrations sh wn in Table 18, then mixed with ^{125}I-PDGF-BB and 10 μg/ml heparin (Elkins-Sinn, Inc.), and 1 ml aliquots of the mixture were added in triplicate to wells of BO54 cells. On a separate plate, the same dilution series of. 163.31
20   was added to ^{125}I-PDGF-BB without heparin. A set of triplicate wells on each plate contained ^{125}I-PDGF-BB plus 1 μg/ml unlabeled PDGF-BB to determine the level of nonspecific binding by ^{125}I-PDGF-BB. The plates were kept on ice while samples were being added, then
25   incubated for 1.5 hour at 4°C on a rotary shaker. After washing 3x with PBS, the cells were incubated with an extraction buffer, and the extracts were transferred t 12 x 75 mm tubes and counted in a gamma counter.

          The results, presented in Table 18, demonstrate
30   that the presence of heparin had no significant effect on dose-dependent inhibitory activity of MAb 163.31 on ^{125}I-PDGF-BB binding to BVSMCs. Thus, the combinatorial effectiveness of coordinately administering heparin with the anti-PDGFr antibodies, shown in the above examples,
35   d es not appear to be attributable to any direct modulation by heparin of the PDGF-BB binding inhibit ry activity f the anti-PDGFr-beta antibody.

Table 18
Inhibition of ^{125}I-PDGF-BB binding to Smooth Muscle Cells By MAb 163.31 in the Presence and Absence of Heparin

	Mean Specific cpm ± S.D. (percent of control)	
MAb 163.31	**(+) Heparin**	**(-) Heparin**
24 μg/ml	888.0 ± 162.6 (39.5%)	715.0 ± 70.0 (36.6%)
8 μg/ml	911.0 ± 291.3 (40.5%)	944.0 ± 89.1 (48.3%)
2.7 μg/ml	1,176.0 ± 107.5 (52.3%)	1,021.0 ± 27.0 (52.2%)
0.9 μg/ml	1,221.0 ± 192.9 (54.3%)	1,136.0 ± 29.8 (58.1%)
0.3 μg/ml	1,549.0 ± 173.5 (68.9%)	1,354.0 ± 46.4 (69.3%)
0.1 μg/ml	1,682.0 ± 104.7 (74.8%)	1,687.0 ± 6.0 (86.3%)
0.033 μg/ml	2,174.0 ± 236.1 (96.7%)	1,726.0 ± 92.4 (88.3%)
0.0 μg/ml	2,248.0 ± 110.3 (100.0%)	1,955.0 ± 24.0 (100.0%)

Example 24
Continuous Intravenous Infusion of MAbs 169.31 and 163.31 into Baboons and Analysis of the Baboon Anti-Murine IgG Response

This study was designed to monitor circulating levels of the murine anti-PDGFr antibodies following continuous infusion by either intravenous or intraperit n al routes. A pool of anti-PDGFr MAbs 163.31 and 169.31 was made with the two antibodies at

approximat concentrati ns f 36 and 22 mg/ml, respectively. Th antibodies were f rmulated in a physiologically acceptable carrier of 1.5% glycine, 0.2M NaCl, and 0.01% Tween-20. The antibody pool was l aded

5 into Alzet 14 day osmotic pumps, which contain 2.1 ml f sample and deliver at a rate of approximately 5 µl/hour. Two pumps were placed into each of three experimental animals. Two of the animals had the pumps placed intraperitoneally (IP) into the peritoneal cavity, and

10 one animal had the pumps placed into the subcutaneous space and the antibody delivered intravenously (IV) by the use of a silastic catheter placed into the venous system.

Plasma samples were collected from the

15 experimental animals at 1, 7, 14, 21, and 28 days following pump placement. The plasma samples were analyzed for circulating levels of the anti-PDGFr antibodies by ELISA. In addition, the plasma samples were analyzed for the presence of baboon antibodies

20 directed towards the murine antibodies.

To analyze for the presence of the murine antibodies, 96-well microtiter plates were coated with either goat anti-mouse IgG1 (Sigma Chemical Co.) or goat anti-mouse IgG2a (Boehringer-Mannheim, Indianapolis, IN)

25 at 1 µg/ml in ELISA A buffer. The plates were incubated overnight at 4°C, then washed 2x with ELISA C buffer. The plates were blocked by the addition of ELISA B buffer for 2 hours at 37°C, then washed 2x with ELISA C buffer. The baboon plasma samples were diluted with ELISA B

30 buffer, then added to the appropriate test w lls. Dilutions of purified monoclonal antibodies 163.31 and 169.31, diluted in control baboon plasma were added to a set of test wells in order to generate a standard curve for use in quantifying antibody levels in the baboon

35 plasma samples. The test antibody samples were incubated for 2 h urs at 37°C, then th wells were washed 3x with ELISA C buffer. Goat anti-mouse IgG conjugated with

h rseradish perixidase (Tago, Burlingame, CA) was then added t the wells, and the plates were incubated at 37°C for an additional 2 hours. The wells were washed with ELISA C buffer, then incubated with Reaction Buffer for approximately 1 minute. The reaction was stopped by the addition of 1N H_2SO_4, and the plates were read in an ELISA microtiter plate reader at 492 nm. Using the values obtained from the purified antibody sampl s, a standard curve was generated for each antibody, and the concentrations of the antibodies in the baboon plasma samples were determined from these curves.

The results, presented in Table 19, demonstrate that there was a peak in the circulating antibody levels at 1 day following pump placement in the i.v. infused animal, while the peak antibody levels were found at day 7 in the intraperitoneal infused animals. At days 14, 21 and 28 the circulating antibody levels were less than 1% of the peak levels measured at the earlier time points.

Table 19
Circulating Levels of Anti-PDGFr Antibody Following Continuous Infusion Into Baboons

| Day | Animal A (IP) | | Animal B (IP) | | Animal C (IV) | |
	MAb163	MAb 169	MAb163	MAb169	MAb163	MAb169
1	1000	1600	340	600	8000	6400
7	9000	7000	4000	3200	2500	200
14	14	64	5	6	8	40
21	8	50	5	8	4	18
28	7	40	1	2	3	14

Data are presented as ng/ml of circulating levels of the anti-PDGFr MAbs in baboon plasma at various times following the initiation of antibody infusion. IP: Intraperitoneal infusi n, IV: Intravenous infusion.

Studies t measure p tential baboon antibody generated against the infused murine antibodies were performed using ELISA. The results suggested that the low levels of circulating antibody measured at 14 days were due to an immune response generated in the baboons against the murine antibodies. These findings sugg st that to provide a sustained level of circulating anti-PDGFr antibody in a primate system, it is useful to select antibodies so as to minimize their immun genic potential. This can be done by a variety of conventional means, including by making Fab or F(ab')2 fragments, r by constructing mouse/human chimeric antibodies or other antibody fragments or constructs with decreased immunogenicity.

Example 25
Determination of Circulating Half-Life of Chimeric Anti-PDGFr-Beta Antibody in Cynomolgus Monkeys

Metabolism of the chimeric anti-PDGFr-beta antibody was determined in Cynomolgus monkeys. This was achieved by use of ^{125}I-labeled antibody. Purified antibody was labeled with ^{125}Iodine by the chloramine T method to a specific activity of approximately 10 μCi/μg of antibody. Three male Cynomolgus monkeys were used in this study, ranging in body weight from 6.0 to 6.6 kg. On the morning of the experiment the radiolabeled antibody was drawn into a 3 ml syringe and placed in an infusion pump. The monkeys were anesthetized with ketamine, and the saphenous veins were cannulated with a 24 gauge intravenous catheter (SURFLO, Terumo Medical Corp., Elkton, MD) attached to polyethylene tubing. The syringe containing the antibody was attached to the tubing, and the infusion was begun at a rate of 0.5 ml/minute, followed by a 0.5 ml flush with saline. Each

animal received a dose f 3.18 mg f antibody/kg body weight.

Immediately following the infusion, blood sampling was begun. In order to determine the half-life of the antibody, samples were drawn into EDTA-containing vacutainer tubes at 0, 0.083, 0.25, 0.5, 1, 2, 4, 8, 12, 24, 72, 120, 168, 240, 336, 504, and 672 hours. Blood was spun, plasma was decanted, and radioactivity was determined in a gamma counter (80% efficiency). Antibody concentrations in the plasma were determined by comparing the counts in the blood to the initial specific activity of the radiolabeled antibody, taking into account the decay rate for ^{125}I. Analysis of the antibody concentrations in the plasma samples showed the half-life of the antibody to be approximately 50 hours.

Example 26
Development of a Sequential Arterial Injury Model in Baboons for Testing Antihyperplastic Agents and Treatments Following Vascular Injury

A model of sequential arterial injury was developed in the baboon to allow testing of the anti-PDGFr antibodies for their ability to inhibit experimentally induced intimal hyperplasia in primates. This model was designed to allow each animal to act as its own control by utilizing bilaterial arterial injuries introduced at 28 day intervals.

Baboons weighing approximately 10 kg each were used in this study. The initial surgical procedure closely resembled the vascular reconstructive procedure of balloon angioplasty used in clinical applications for treatment of human atherosclerosis. For each animal, an initial balloon denudation pull-back injury was made to the saph nous artery. On day 28 the animals underwent a second surgical proc dure whereby the initially injured artery was excised, and the excised artery was perfusion-

fixed *ex vivo* under 100 mm Hg pressure for 1 hour with a 10% formalin solution. Following excision of the first artery, the contralateral saphenous artery received a ballon denudation injury. Following the second 28-day

5   period the second injured artery was excised and perfusion fixed ex vivo in a similar manner as the first artery.

Both of the excised arteries were separated into multiple sections and embedded in paraffin.

10   Sections were cut from multiple tissue blocks, stained with hematoxylin and eosin, then analyzed by morphometric analysis for intimal lesion formation using a computerized image analysis system (Ferns et al., Science 253:1129, 1991).

15   The results of the study, presented in Table 20, demonstrate that the intimal/medial ratios (I/M) for the contralateral arteries were very similar, even though the initial arterial injuries were made 28 days apart. This suggests that the presence of an initial arterial

20   injury, and the subsequent removal of the injured arterial segment, does not effect the response of the second injured artery. These results also demonstrate that the extent of arterial injury, as measured by the intimal/medial ratio, is more similar within an animal

25   than between animals. These findings demonstrate that it is possible to utilize a sequential injury model, whereby each animal acts as its own control, for evaluating the efficacy of therapeutic compounds to inhibit intimal lesion formation in the baboon.

Table 20
Analysis of Intimal Lesion Development Following
Sequential Balloon Denudation Arterial Injuries in the
Baboon

Animal	Side	Intimal Area (mm^2)	Medial Area (mm^2)	I/M Ratio
Z6	R	0.1256	1.085	0.1151
Z6	L	0.0543	0.547	0.0995
Z8	R	0.1877	1.269	0.1493
Z8	L	0.1125	0.826	0.1344

Example 27
Evaluation of Anti-PDGF Receptor Antibody/Heparin Therapy
to Inhibit Intimal Hyperplasia in Primates

Baboons weighing approximately 10 kg each are used to study the efficacy of anti-PDGFr MAbs and heparin. An initial balloon denudation pull-back injury is made to one saphenous artery of the animal. Additional arterial beds, such as the radial, brachial, carotid or femoral arterial beds, may also be used. At the time of injury a femoral vein catheter and sub-cutaneous (SQ) osmotic pump are inserted (twenty-eight day pumps, two pumps per animal). The pumps deliver a combined rate of 5 μl/hour. During the first 28-day control period the pumps are loaded with placebo saline solution.

During the 28-day period following the balloon injury, the animals receive i.v. injections of placebo buffer. Typical injection times are 0, 3, 7, 14 and 21 days following the initial injury.

On day 27 one-half of the animals receives three intramuscular (IM) injections, 8 hours apart, of

bromodeoxyuridine (Brdu) to all w f r labeling f replicating cells in the artery wall.

On day 28 a second surgical procedure is performed whereby the previously injured artery is

5 excised and perfusion-fixed under 100 mm Hg pressur for 1 hour with a 10% formalin solution. Following excision of the first artery, the contralateral saphenous artery receives a ballon denudation injury. Femoral vein catheters and SQ osmotic pumps are then inserted. Th

10 pumps are loaded with unfractionated heparin (Elkins-Sinn, Inc.) to be delivered at the rate of approximately 0.1 mg/kg/hour. Circulating levels of heparin are monitored using a commercially available kit (American Diagnostica).

15 The animals receive an i.v. injection of mouse/human chimeric anti-PDGF receptor antibody one hour prior to the second balloon injury, followed by i.v. injections at days 3, 7, 14 and 21 post-angioplasty. A dose of 10 mg/kg of the anti-PDGF receptor antibody is

20 used. to give an initial circulating level of 350 μg/ml and a level of approximately 20 μg/ml at 7 days (based on a T$_\frac{1}{2}$ for the chimeric antibody of approximately 50 hours in cynamolgus monkeys).

Blood draws are done on a weekly basis at the

25 time of antibody injection to determine the circulating levels of anti-PDGF receptor antibody and circulating levels of heparin. Circulating antibody levels are determined using an ELISA. Heparin levels are determined as described above. In addition, animals are monitor d

30 for any baboon antibody response directed towards the chimeric antibody. If a positive serum titer is detected, the antisera are measured for neutralizing activity towards the chimeric antibody.

Following the second 28-day period, the second

35 injured artery is excised and perfusion-fixed ex vivo in a similar manner as the first artery. In addition, those animals n t treated with Brdu at the time of the excision

of the first artery ar now treated with bromod oxyuridine to label replicating cells in the artery wall as was done at the time of harvesting the control arteries.

Both the control arteries and the arteries harvested following antibody treatment are separated int multiple sections and embedded in paraffin. Sections are cut from multiple tissue blocks and stained with hematoxylin and eosin, then analyzed by morphometric analysis for intimal lesion formation using a computerized image analysis system (Ferns et al., Science 253: 1129, 1991) Separate sections are stained with an anti-Brdu antibody (Boehringer-Mannheim, Inc.) to determine the level of replicating cells in each tissue section.

Preliminary studies, disclosed above, using the sequential injury model demonstrated that there was low variability between the arteries injured 28 days apart. Analysis of preliminary studies suggested that an n = 15 would be required to observe a 50% decrease in lesion development with a 95% confidence limit.

Data obtained from the multiple sections from each artery are pooled to obtain a single lesion number for each artery. The control and test artery for each animal are compared in a paired format to determine the significance of antibody treatment to decrease intimal lesion development. Similar analysis is used to measure the level of intimal and medial cell replication rates, determined by staining for Brdu.

A similar protocol is used for evaluation of a pooled anti-PDGF alpha receptor (MAb 169.3.1) and anti-PDGF beta receptor (MAb 163.3.1) antibody preparation, as well as for the two chimeric antibodies individually.

The protocol described above can be used to evaluate th anti-PDGF receptor antibodies individually, in c mbinati n, or in the the presence or absence of various doses of heparin. In addition to using

morphom tric analysis t lo k f r changes in intimal hyperplasia, additi nal types f analysis that can be used to monitor lesion formation include angiography, intravascular ultrasound, and nuclear magnetic res nance
5   scanning.   In addition, tissue samples can be obtained from the site of injury at multiple time points following the induction of the injury by reduction athectomy.

Although the foregoing invention has been
10   described in some detail by way of illustration and example for purposes of clarity of understanding, it will be evident that certain changes and modifications may be practiced within the scope of the appended claims.